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13. Abstract: The key goal of this project is to test the hypothesis that exposure to mixtures of the test compounds, PB (oral), DEET (dermal), and permethrin (dermal) reduces the body's ability for their detoxification, resulting in increased bioavailability of the parent compounds and of their enhanced delivery to the toxicity site(s), leading to pronounced toxicity. The results reported in this Annual Report support this hypothesis as follows: 1. Although exposure to single compounds caused locomotor and sensorimotor performance deficit in rats, combined exposure resulted in greater deficit even at one-tenth of real-life exposure doses. 2. Combined exposure to the three test compounds with stress increased the permeability of the blood-brain barrier (BBB) as shown by the uptake of [³ H]hexamethonium iodide, penetration of horseradish peroxidase and immunostaining for endothelial-barrier antigen (EBA), and inhibition of brain acetylcholinesterase. 3. Exposure to DEET and/ or permethrin for 60 days, and PB, DEET, and permethrin in combination with stress for 28 days caused neuronal cell death in cerebral cortex and hippocampus, and Purkinje cells in the cerebellum. These alterations are consistent with the neurobehavioral changes and breakdown of the BBB. 4. Combined exposure to chemicals and stress caused liver damage and decreased detoxification of test compounds. 5. Combined exposure increased the bioavailability of test compounds because liver damage and because of competition for the diminished amount of available enzyme(s) in the plasma and liver. These results are consistent with recent DOD report that Gulf war veterans are prone to neurologic diseases such as amyotrophic lateral sclerosis (ALS).			
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FOREWORD

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Principal Investigator's Signature

10/21/02
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Introduction

A) The Nature of the Problem

The goal of this project is to elucidate the mechanisms of interactive effects of combined exposure to the anti-nerve gas prophylaxis, pyridostigmine bromide (PB); the insect repellent, DEET; and the insecticide, permethrin. The studies were carried out using doses low enough to be representative of human exposure levels. The hypothesis to be tested is that combined exposure to chemicals, impedes the body's ability for their detoxification, resulting in increased concentration of the parent compounds in circulation and of their enhanced delivery to the toxicity site, leading to increased toxicity. The studies have been carried out by a tiered approach using single compounds, as well as binary and tertiary combinations of test chemicals. Animals have been undergoing behavioral evaluations and at termination, brain and blood esterases are determined and the integrity of the blood brain barrier is evaluated. Pharmacokinetics and metabolism of test compounds, alone and in combination are carried out.

B) Approach

Our hypothesis is that interactions between combined chemical exposure would result in greater toxicological and pathological changes, and certain environmental modifying factors such as stress may exacerbate the toxic effects in combined exposure scenario. In order to investigate these toxic interactions we have utilized a various experimental modalities. The experimental approaches include neurobehavioral, neurochemical, pathological, and analytical methodologies. We have carried out dose-response studies ranging from 0.1-10 x the estimated human exposure of PB, DEET, and permethrin. In these studies we carried out neurobehavioral as well as neurochemical assessment following exposure to a single or multiple chemicals. Blood-brain barrier (BBB) permeability was evaluated by brain uptake of [³H]hexamethonium iodide uptake as well as immunohistochemical staining for horseradish peroxidase. In the pathological studies, we have evaluated the extent of neurodegeneration within the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of hippocampus, and the cerebellum following dermal application of DEET and permethrin, alone and in combination. Additionally, we studied the effect of one environmental modifier, stress on the neurotoxicity associated with concurrent exposure to PB, DEET and permethrin. The pathological changes were studied by immunohistochemical evaluation of microtubule-associated protein-2 (MAP-2), glial fibrillary acidic protein (GFAP), and microglial activation by lectin binding. Furthermore, we evaluated the possible mechanism(s) of neurotoxic effects of single or combined exposure by assessing the permeability of blood-brain barrier (BBB) and the involvement of biomarkers for reactive oxygen species (ROS).

In order to perform comprehensive pharmacokinetics and bioavailability studies, we established the analytical methodology for simultaneous evaluation of PB, DEET, and permethrin, and their metabolites by HPLC. Bioavailability of test compounds, PB, DEET, and permethrin was evaluated under the following conditions: a). Oral

bioavailability of PB, alone or when administered concurrently with dermal DEET and/or dermal permethrin. b). Dermal bioavailability of DEET, alone or when administered concurrently with oral PB and/or dermal permethrin. c). Dermal bioavailability of permethrin, alone or when administered concurrently with oral PB and/or dermal DEET.

Background

CNS cyto-architecture is maintained by a complex cellular milieu that involves neurons and a variety of cells of astrocytic and glial origin. In order for the CNS to function properly and to respond to external stimuli, it is absolutely required that a proper communication is maintained within these cells. A major determinant of neuronal morphology is the cytoskeleton. Different components of cytoskeleton within the neurons and astrocytes provide forces to maintain the appropriate cellular structure, e.g. neuronal dendrites and axons are maintained in stable conditions by the force provided by the elements of cytoskeleton (1). Such interactions are essential for proper synapse formation. The components of cytoskeleton are microfilament, intermediate filaments and microtubules. An important neuronal component, MAP-2 is enriched in dendrites and cell bodies (2), in which it stabilizes the polymerized tubulin. Abnormal regulation of expression of MAP-2 causes suppression of neurite outgrowth and reduction in number of neurites in cultured neurons (3). Similarly, aberrant intermediate filament proteins have been linked to diseases of neurodegeneration (4). A major component of astrocytic intermediate filament, GFAP is upregulated in response to reactive gliosis as a consequence of a variety of insults, such as exposure to neurotoxic chemicals, trauma, and neurodegenerative diseases that affect the CNS (5). The function of GFAP is not well understood, but it has been suggested to play important role in long term maintenance of brain cyto-architecture (6), proper function of BBB (7), and modulation of neuronal functions (8).

Microglia are involved in brain function under both normal and pathological conditions (9, 10). In normal brain, resident ramified/resting microglia are activated to become rod-shaped or amoeboid shape in response to injury or toxic insult. Activated microglia proliferate, engulfing degenerating elements (11, 12), while secreting cytotoxic agents that induce neuronal death and demyelination (13, 14).

Oxidative stress resulting from environmental toxicants has been considered as a cause of chemical exposure related diseases. In the CNS, oxidative DNA damage has been observed following ischemia (15). Increased formation of 8-hydroxy-2'-deoxyguanosine has been reported in response to exposure with environmental toxicants (16, 17). Thus, it is apparent that a diverse mechanism could be activated in response to toxic insult that may result in pathological changes.

Test Compounds:

In our studies, we chose to study PB, DEET, and permethrin because our previous results have demonstrated that a combination of these chemicals causes greater neurobehavioral

and neurotoxic changes than each chemical alone (18, 19, 20). In addition, thousands of U.S. Army personnel were presumably exposed to a combination of these chemicals during Persian Gulf War, and therefore, these studies may have human relevance.

Pyridostigmine Bromide (PB): PB is a quaternary dimethyl carbamate that has been used for the treatment of myasthenia gravis (21). It was given to veterans for prophylactic protection to shield acetylcholinesterase (AChE) from the nerve agent poisoning by reversibly inhibiting 30-40% of the AChE in the peripheral nervous system, thus protecting the enzyme from irreversible inhibition by nerve agents (22). The enzyme activity is restored following spontaneous decarbamylation resulting in near normal neuromuscular and autonomic functions (22). Toxic symptoms associated with PB overdose are primarily associated with over-stimulation of nicotinic and muscarinic receptors in the peripheral nervous system resulting in exaggerated cholinergic effects such as muscle fasciculations, cramps, weakness, muscle twitching, tremors, respiratory difficulty, gastero-intestinal tract disturbances and paralysis (23). The major metabolic product of PB is 3-hydroxy-*N*-methylpyridinium resulting from the carbamate hydrolysis that abolishes its cholinergic action (24, 25). Central nervous system effects of PB are not observed unless blood-brain barrier (BBB) permeability is compromised, because PB does not cross the BBB owing to the positive charge on the quaternary pyridinyl nitrogen (26).

***N,N*-diethyl-*m*-toluamide (DEET):** DEET is commonly used as an insect repellent against mosquitoes, flies, ticks and other insects in the form of lotion, stick or spray (27, 28). Extensive and repeated topical application of DEET resulted in human poisoning including death (29, 30, 31). The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty in speech, seizures, impairment of cognitive function and coma (28). Extremely high levels of DEET exposure has been reported to cause spongiform myelinopathy (32). Because DEET efficiently crosses the dermal barrier (33, 34) and localizes to dermal fat deposits (35, 36), it is possible that DEET may enhance the availability of drugs and toxicants in other organs and cause regulatory changes such as changes in blood brain permeability. However, it is not known with certainty that DEET could enhance the neurotoxicity associated with permethrin or PB because of its lipophilic nature.

Permethrin: Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers (37). Its insecticidal activity persists for several weeks following a single application. Permethrin intoxication results as a consequence of modification of sodium channel such that it remains open for a longer time, leading to repetitive discharges after single stimulus (38). This repetitive nerve action is associated with tremors, hyperactivity, ataxia, convulsions, and in some cases to paralysis. It has been reported that high dose of permethrin given to rats induced peripheral nerve damage through its effects on sodium channels (39).

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BODY

Key Research Accomplishments

The goal of this project is to characterize and elucidate interactive effects of combined exposure to the anti-nerve gas agent prophylaxis, pyridostigmine bromide (PB); the insect repellent, DEET; and the insecticide, permethrin. This goal is to be accomplished through carrying out the following studies:

- A. Neurotoxic effects of PB, DEET, and permethrin, alone and in combination
- B. Effect of the test compounds in the permeability of the blood brain barrier
- C. Bioavailability, Pharmacokinetics and Metabolism of test compounds

During the past period, we have carried out the following studies to accomplish the project's objectives.

1. Effect of dermal application of DEET and/or permethrin on brain neuronal structure in male rats
2. Effect of dermal application of DEET and permethrin, alone and in combination on the release of rat brain mitochondrial cytochrome c
3. Effect of Pyridostigmine bromide, DEET, and/or permethrin on urinary excretion of 3-nitrotyrosine, a marker for oxidative stress
4. Effect of DEET and permethrin, alone or in combination on the urinary excretion of 6 β -hydroxycortisol, a marker for hepatic CYP450 induction rats
5. Pharmacokinetic interactions between DEET (*N,N*-diethyl-*m*-toluamide) and permethrin following dermal administration in rats

1. Effect of Daily Dermal Application of DEET and/or Permethrin on Brain Neuronal Structure in Male Rats

ABSTRACT:

To determine the effect of sub-chronic dermal application of these chemicals on the adult brain, we evaluated histopathological alterations in the brain of adult male rats following a daily dermal dose of DEET (40 mg/kg in 70% ethanol) or permethrin (0.13 mg/kg in 70% ethanol) or a combination of the two for 60 days. Control rats received a daily dermal dose of 70% ethanol for 60 days. Animals were perfused and brains were processed for morphological and histopathological analyses following the above regimen. Quantification of the density of healthy (or surviving) neurons in the motor cerebral cortex, the dentate gyrus, CA1 and CA3 subfields of the hippocampus, and the cerebellum revealed significant reductions in all three treated groups compared to the control group. Further, animals receiving either DEET or permethrin exhibited a significant number of degenerating (eosinophilic) neurons in the above brain regions. However, degenerating neurons were infrequent in animals receiving both DEET and permethrin, suggesting that, in animals receiving combined DEET and permethrin, neuronal cell death occurs earlier than animals receiving either DEET or permethrin alone. The extent of neuron loss in different brain regions was similar between the three treatment groups except the dentate gyrus where neurodegeneration was significantly greater with exposure to DEET alone. The neuron loss in the motor cerebral cortex and the CA1 subfield of all treated groups was also corroborated by a significant decrease in microtubule associated protein-2 immunoreactive elements (15-52% reduction) with maximal reductions occurring in rats receiving DEET alone; further, the surviving neurons in animals receiving both DEET and permethrin exhibited wavy and beaded dendrites. Analysis of glial fibrillary acidic protein immunoreactivity revealed significant hypertrophy of astrocytes in the hippocampus and the cerebellum of all treated groups (24-106% increase). Thus, sub-chronic dermal application of DEET and permethrin to adult rats, alone or in combination, leads to a diffuse neuronal cell death in the cerebral cortex, the hippocampal formation, and the cerebellum. Collectively, the above alterations can lead to many physiological, pharmacological, and behavioral abnormalities, particularly motor deficits and learning and memory dysfunction.

INTRODUCTION

During the Persian Gulf War (PGW) in 1991, many service personnel were exposed to a variety of chemicals, including DEET and permethrin [2, 18]. The chemicals, DEET and permethrin, in particular, were used extensively by service personnel as a protection against insect-born diseases [18]. In the last decade, many veterans have complained of chronic symptoms including headache, loss of memory, fatigue, muscle and joint pain, and ataxia. DEET and permethrin have been implicated as one of the likely neurotoxic agents that may have played a significant role in the occurrence of neurological disorders in some veterans of the PGW.

The insect repellent *N, N*-diethyl *m*-toluamide (DEET) and the pyrethroid insecticide, permethrin (3-phenoxybenzyl [±]-cis, trans3- [2,2-dichlorovinyl]-2,2-dimethylcyclopropane-1-carboxylate) have been used extensively by humans since their introduction. DEET is used as an effective repellent against mosquitoes, flies, ticks and other insects in the form of lotion, stick or spray [21, 28]. Extensive and repeated topical application of DEET can result in human and animal poisoning including death [10, 15,20,29]. The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function and coma [21]. High levels of DEET exposure have been reported to cause spongiform myelinopathy [38]. DEET efficiently crosses the dermal barrier [17,34,40,] and localizes in dermal fat deposits [5,32]. Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers [8]. It provides insecticidal activity for several weeks following a single application. Permethrin toxicity is due to prolonged opening of the sodium channels, leading to repetitive discharges after a single stimulus [23]. This repetitive nerve action is associated with tremors, hyperactivity, ataxia, and convulsions and in some cases, paralysis [30].

The majority of the symptoms reported by the affected veterans of the PGW involve abnormal regulation of functions in either the central or peripheral nervous systems or both. Recent studies in our laboratory have suggested significant sensorimotor deficits and blood-brain barrier disruption following exposure to DEET and permethrin [1]. In this study, we evaluated the extent of neurodegeneration within the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the cerebellum of

adult rats after daily dermal application of DEET and permethrin for 60 days, alone or in combination. Dermal doses of 40mg/kg DEET and 1.3mg/kg permethrin were applied daily, because they were determined to be the doses that military personnel were exposed to during the Persian Gulf War (Dr. W. C. McCain, US Army Center for Health, Promotion, and Prevention Medicine, Aberdeen Proving Ground, MD; Personal communication).

Following exposure of animals to DEET and permethrin for 60 days, alone or in combination, we rigorously quantified neurodegeneration in the above brain regions by several indices. This includes: (i) measurement of the density of both healthy (surviving) and dying neurons; (ii) quantification of the reductions in the microtubule associated protein-2 (MAP-2) immunoreactive elements; and (iii) measurement of the extent of up-regulation in the glial fibrillary acidic protein (GFAP) immunopositive structures. In addition, we investigated the histopathological alterations in surviving neurons, particularly the orientation and cytoarchitecture of dendrites using MAP-2 immunostaining.

MATERIALS AND METHODS

Chemicals and antibodies

DEET (97.7%, *N, N*-diethyl *m*-toluamide) was purchased from Sigma Chemical Co., St. Louis, MO. Technical-grade permethrin (+/-)-*cis/trans*-3- (2,2-dichloroethyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester (93.6%) was obtained from Roussel Uelaf Corporation (Pasadena, TX). The monoclonal antibody (SMI 52) against microtubule-associated protein-2 (MAP-2) was from Sternberger Monoclonals (Lutherville, MD), the polyclonal antibody against glial fibrillary acidic protein (GFAP) was from Dako (Dako Labs, Carpinteria, CA), and the avidin-biotin-complex (ABC) detection kits were purchased from Vector Labs (Burlington, CA). All other chemicals and reagents were of highest purity available from commercial sources.

Animals

Male Sprague-Dawley rats (200-250gms) obtained from Zivic Miller, Allison Park, PA were used. Animals were randomly assigned to control and treatment groups of five rats (n=5) and housed at 21-23°C with 12-hr light/dark cycle. They were supplied with Purina Certified Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. The rats were allowed to adjust to their environment for a week before treatment. Animal care was in accordance with the Army Guidelines and Duke University Animals Care and Use Committee.

Dermal application of DEET and permethrin

The chemicals (DEET and permethrin) were applied directly to the skin of pre-clipped area (2.5 cm²) in the back of the neck to give the desired concentration of test compounds in 1ml of the vehicle solution. Groups of five rats received a daily dermal dose of 40 mg/kg DEET in 70% ethanol or 0.13 mg/kg permethrin in 70% ethanol, or the combination of DEET and permethrin. Control animals received an equal volume of the vehicle. The treatment was carried out daily, 7days a week, for 60 days. The doses of DEET and permethrin are based on an estimate of the exposure that may have occurred to army personnel during Gulf War [1]. For combined exposure, both chemicals (at the single dose level) were applied simultaneously on adjacent areas of the skin in the back of the neck.

Histopathological assessment

Twenty-four hours after the last dose, animals belonging to each group (n = 5 per group) were anesthetized with pentobarbital (100 mg/kg) and perfused through the heart with saline followed by 4% paraformaldehyde and 0.1% gluteraldehyde in Tris buffer. The brains were removed, post-fixed, and embedded in paraffin. Four-micrometer thick coronal sections were cut through different brain regions. In every brain, representative sections (n = 5) through the motor and sensory cortex, the septal hippocampus, and the cerebellum were processed and stained with hematoxylin and eosin (H&E) for light microscopy.

Microtubule associated protein-2 (MAP-2) and glial fibrillary acidic protein (GFAP) immunohistochemistry

Sections were deparaffinized and blocked with 10% normal serum (normal horse serum for MAP-2, and normal goat serum for GFAP) in 0.05M TBS for 30 minutes. Sections were incubated for overnight at room temperature in primary antisera diluted at 1:1000 for MAP-2 in 0.05M Tris buffer saline (TBS) containing 1% normal horse serum, and 1:10,000 for GFAP with 0.05M TBS containing 1% normal goat serum. Following a thorough rinse in 0.05% TBS, sections were incubated for 1 hr at room temperature in appropriate biotinylated secondary antibody (i. e., horse-anti-mouse IgG for MAP-2, and goat-anti-rabbit IgG for GFAP, diluted 1:200) containing 1% normal serum (horse serum for MAP-2 staining and goat serum for GFAP staining).

Sections were rinsed with several changes of 0.05M TBS and incubated for 1hr in the avidin-biotin peroxidase complex solution diluted 1: 25 in 0.05M TBS. Following this, the sections were rinsed with several changes of 0.05M TBS and incubated with 3,3-diaminobenzene tetrahydrochloride (DAB) for 10 min. The reaction was stopped by several rinses in 0.05M TBS. The sections were then dehydrated in alcohol, cleared in xylene and cover slipped with permount.

Quantitative evaluation of the number of healthy and dying neurons in different brain regions:

The numerical density of healthy (surviving) and dying neurons per mm² area of tissue in H & E stained sections was measured for layers III and V of the motor cortex, granule cell layer of the dentate gyrus, pyramidal cell layer of CA1 and CA3 subfields of the hippocampus, and Purkinje cell layer of the cerebellum in lobule 2 of the cerebellar vermis and crus 2 ansiform lobule of the cerebellar hemisphere. Five sections through each of the above brain regions were employed for measurements in each animal belonging to the following four groups: (a) control animals (n = 5); (b) animals treated with DEET (n = 5); (c) animals treated with permethrin (n = 5); and (d) animals treated with both DEET and permethrin (n = 5). Measurements in sections from various groups were performed in a blinded fashion using

experimental codes. The coding was such that animal treatments were not known during measuring; however, sections that came from the same animal were identified. All measurements were performed using a Nikon E600 microscope equipped with eyepiece grid. At a magnification of 400X (using 40X objective lens and 10X eyepieces), both dying and healthy neurons, within a unit area of each section were counted.

The unit area selected for measurements varied for different regions of the brain, depending on the availability of the overall area for different layers. The area measured was 0.019 mm² for layer III of the motor cortex, 0.063 mm² for layer V of the motor cortex, 0.013 mm² for the dentate granule cell layer, 0.0063 mm² for the CA1 pyramidal cell layer, 0.013 mm² for the CA3 pyramidal cell layer, and 0.0063 mm² for Purkinje cell layer of the cerebellum. For measurement of the surviving neurons, only those which exhibited hematoxylin stained nucleus with a clear nucleolus were counted. For measurement of dying neurons, only those neurons that exhibited dense eosinophilic staining in both soma and proximal dendrites were counted. Finally, the density of neurons per unit area was transformed to the numerical density per mm² area of respective brain region.

The mean value for each of the six brain regions (layers III and V of the motor cortex, granule cell layer of the dentate gyrus, CA1 and CA3 pyramidal cell layers of the hippocampus, and Purkinje cell layer of the cerebellum) was calculated separately for each animal by using data from 5 sections before the means and standard errors were determined for the total number of animals included per group. Mean values between different groups of animals were compared separately for each of the above brain regions using one-way ANOVA with Student's Newman-Keuls multiple comparison post-hoc test.

Morphometric analyses of MAP-2 positive and GFAP positive immunoreactivity in different brain regions:

Morphometric analyses of MAP-2 positive and GFAP positive immunoreactive structures in different regions were performed by using Scion Image for Windows, based on NIH Image for Macintosh (Scion Corporation, Frederick, MD). For every brain region, two sections were measured in each animal. All

data were collected blind to experimental codes and means were calculated for each animal individually before the means were determined for the five animals per group. Statistical comparisons on MAP-2 and GFAP measurements in different brain regions between control and treated groups utilized ANOVA with Student's Newman-Keuls multiple comparisons post-hoc test.

The area occupied by MAP-2 positive immunoreactive structures per unit area of tissue (0.044 mm^2 in area) was determined for layers III and V of the motor cerebral cortex, and CA1 subfield of the hippocampus. The area occupied by GFAP positive immunoreactive structures per unit area of tissue (0.0176 mm^2 in area) was determined in layer V of the motor cerebral cortex, the dentate gyrus, and CA1 and CA3 subfields of the hippocampal formation, and the white matter of the cerebellum. For every region, the microscopic image using 20X objective lens was transferred to the computer screen by focusing the appropriate area of the immunostained section with a Nikon E600 microscope equipped with a digital camera (DAGE-MTI, CCD100) connected to an IBM Computer. The same intensity of light in the microscope and the same parameters in the digital camera were used to digitize all the samples from different brain regions. Images in the Scion Image are two-dimensional arrays of pixels (picture elements). Pixels are represented by 8-bit unsigned integers, ranging in value from 0-255. Scion Image displays zero pixels as white and those with a value of 255 as black. The background and target values were set to 145 and 255, respectively, following digitization of the original gray value image in the computer screen. These values were determined by selecting the background and target areas in several sections from both control and treated animals before commencing the measurements on coded slides for statistical analysis. This scale eliminated the background staining completely and retained all the target (MAP-2 or GFAP immunopositive) structures in the range (145-255). The binary image of MAP-2 or GFAP positive elements was then generated by selecting a suitable threshold value (which varied from 155-165) to include all the MAP-2 or GFAP positive structures without any background. The final binary image was crosschecked with the original gray value image by alternating the two images on the computer screen.

Finally, the image was frozen and the area occupied by the MAP-2 or GFAP positive structures in the field was measured by selecting “Analyze particles” command of the Scion Image program. This way, the area of individual particles (i.e. MAP-2 or GFAP immunoreactive structures) in the selected field was measured, and the sum area of all particles was stored for further calculations and statistical analysis. Since spatial calibration of the image was performed in micrometers using “Set Scale” function of the program prior to measurements, the results from area measurements were obtained in square micrometers and converted later into square millimeters.

RESULTS

General Observations

The clinical condition of animals treated with daily dermal application of DEET, permethrin, or the combination of DEET and permethrin was not different from control animals. In addition, statistically, no differences were observed in the weights of animals between the control and treated groups (Fig. 1).

Histopathological Changes

Evaluation of brain sections stained with hematoxylin and eosin (H and E) clearly revealed neuronal degeneration in rats treated with DEET, permethrin, or the combination of DEET and permethrin, in comparison to vehicle treated rats. Degenerating neurons were characterized by eosinophilic staining of both cell body and proximal dendrites. In contrast, the healthy neurons in the same section exhibited hematoxylin stained nucleus (with a clear nucleolus) and eosin stained perinuclear cytoplasm. The brain regions where neuronal degeneration was obvious include the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the Purkinje cell layer of the cerebellum. Other areas of the brain, though showed occasional dying (eosinophilic) neurons in some animals, the overall cytoarchitecture remained comparable to those of control (vehicle-treated) rats. Therefore, detailed investigation of neuropathological alterations using quantitative methods was performed only on the above brain regions.

Alterations in the cytoarchitecture of the motor cerebral cortex

In animals treated with either DEET or permethrin alone, both superficial and deeper regions of the motor cortex exhibited degenerating neurons in H and E stained sections. In superficial region (layers I-III; Fig. 2), degenerating neurons were conspicuous in both layers II and III. Majority of degenerating neurons in these layers were of pyramidal type with prominent eosinophilic apical dendrites (Fig. 2 [A2, A3]). The overall degree of neuronal degeneration was comparable between animals treated with DEET and animals treated with permethrin. In deeper regions of the cortex (layers IV-VI), degenerating neurons were mostly observed in the layer V. These are larger pyramidal neurons with prominent apical and basal dendrites emanating from a larger pyramidal-shaped cell body (Fig. 3 [A2, A3]). The extent of degeneration appeared more with exposure to DEET than with exposure to permethrin. Further, in addition to the presence of many degenerating neurons, both superficial and deeper regions of the cortex in animals treated with either DEET or permethrin exhibited clearly reduced packing density of surviving neurons, in comparison to the cortex of control animals (Figs. 2 [A1-A3] and 3 [A1-A3]. The adjacent sections stained for MAP-2 revealed significantly reduced MAP-2 positive dendrites in layer III and V of animals treated with either DEET or permethrin, in comparison to control animals (Figs 2 [B1-B3] and 3 [B1-B3]). The MAP-2 expression in dendrites also appeared somewhat disrupted and scarcer. Further, immunostaining with GFAP demonstrated hypertrophy of astrocytes with increased GFAP expression in animals treated with either DEET or permethrin compared to control animals (Figs. 2 [C1-C3] and 3 [C1-C3]. Thus, both MAP-2 and GFAP immunostained sections clearly corroborated the DEET and permethrin induced neurodegeneration, as observed in H and E stained samples.

In animals treated with both DEET and permethrin, degenerating (or eosinophilic) neurons were infrequent in both superficial and deeper regions of the cortex (Figs 2 [A4] and 3 [A4]). The packing density of surviving neurons, however, appeared less than that of control animals (Figs 2 [A1, A4] and 3 [A1, A4]). Areas devoid of neurons were conspicuous in layers III and V of the cortex (Figs. 2 [A4] and 3 [A4]). Thus, the lack of degenerating (eosinophilic) neurons in animals receiving both DEET and

permethrin appeared to be due to early cell death of neurons following the combined exposure in comparison to animals receiving either DEET or permethrin alone. The adjacent sections stained for MAP-2 substantiated the above finding by exhibiting reduced MAP-2 staining of dendrites particularly in layer V of the cortex (Figs 2 [B4, C4] and 3 [B4, C4]. The GFAP positive astrocytes were of reactive type and exhibited characteristic GFAP expression in their soma. In addition, the pattern of MAP-2 expression differed from both control animals and animals treated with either DEET or permethrin by showing a lack of expression in soma of neurons, and by their wavy and fragmented appearance in dendrites throughout the thickness of the cortex (Figs. 2 [B4] and 3 [B4]).

Extent of neuron loss, reductions in MAP-2 immunoreactivity, and up-regulation of GFAP immunoreactivity in the motor cortex

Quantification of healthy (or surviving) neurons per mm² area of layers III and V of the motor cortex revealed that animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in the number of surviving neurons in both layers III and V ($p < 0.01$; Fig. 4), in comparison to control animals. Further comparison between treated groups revealed that the extent of reductions in the density of healthy neurons within the motor cortex was similar between the three treatment groups. Analysis of dying neurons revealed that, in layer III of the motor cortex, animals treated with DEET exhibited a significant number of dying neurons, in comparison to control animals ($p < 0.01$; Fig. 4). In layer V of the motor cortex, animals treated with either DEET or permethrin exhibited a significant number of dying neurons compared to control animals ($p < 0.001$ and $p < 0.01$ respectively; Fig. 4). Thus, sub-chronic dermal application of DEET and permethrin, alone or in combination, leads to a significant reduction in the number of surviving neurons in the motor cortex, and the extent of overall reductions in neurons is similar between the three treatment groups. This suggests that concurrent application of DEET and permethrin does not induce enhanced neuron loss in the motor cortex, compared to exposure to either DEET or permethrin.

Quantification of the area of MAP-2 immunoreactive elements per unit area of layers III and V of the motor cortex showed that there were fewer MAP-2 positive structures in the motor cortex of all treated groups (Fig. 5 [A]). In layer III of the cortex, the MAP-2 immunoreactive structures exhibited 27-28% reduction with exposure to DEET or permethrin alone ($p < 0.05$), and 15% reduction with exposure to both DEET and permethrin ($p > 0.05$). Whereas, in layer V of the motor cortex, the MAP-2 immunopositive structures showed 52% reduction with exposure to DEET alone ($p < 0.01$), 35% reduction with exposure to permethrin alone ($p < 0.05$), and 49% reduction with exposure to both DEET and permethrin ($p < 0.01$). The measurement of GFAP immunoreactive structures per unit area of layer V of the motor cortex revealed a significant up-regulation in GFAP positive elements ($p < 0.05$) with exposure to either DEET (74% increase) or permethrin (63% increase; Fig. 5 [B]). However, with combined exposure to DEET and permethrin, there was only a 10% increase in GFAP immunoreactivity ($p > 0.05$).

Alterations in the hippocampal formation

Neuronal degeneration was obvious in the dentate gyrus, and CA1 and CA3 subfields of the hippocampal formation following exposure to DEET and permethrin, alone or in combination. In all treated groups, the thickness and cell packing density of granule cell layer appeared reduced compared to control animals. Further, in dentate gyrus of animals treated with DEET and permethrin, degenerating neurons were observed in both the granule cell layer and the dentate hilus (Fig. 6 [A2-A3]). The GFAP immunoreactivity was enhanced in the molecular layer and the hilus of all three treated groups, in comparison to control animals (Fig. 6 [B1-B4]). The MAP-2 staining of the granule cell layer and the molecular layer did not show any significant differences between animals belonging to different treated groups but appeared slightly reduced compared to the control animals (data not shown).

In CA1 subfield of the hippocampus, the thickness and cell packing density of stratum pyramidale appeared reduced in treated groups compared to control animals (Fig. 7). Further, degenerating neurons were clearly observed in the stratum pyramidale of animals treated with either DEET or permethrin alone

(Fig. 7 [A1-A3]). The MAP-2 staining of adjacent sections demonstrated a conspicuously reduced density of MAP-2 positive dendrites in animals belonging to all three treated groups compared to control animals (Fig. 7 [B1-B4]). In animals treated with DEET, MAP-2 positive apical dendrites in stratum radiatum were thinner and appeared disrupted. Whereas, in animals treated with permethrin, MAP-2 positive dendrites appeared to be either beaded or arranged in aggregates with highly conspicuous vacant spaces between them. In animals treated with both DEET and permethrin, MAP-2 positive dendrites were wavy and thinner. The appearance of MAP-2 staining of apical dendrites in all treated groups highly contrasted with homogenous MAP-2 staining observed in control animals. Immunostaining of neighboring sections for GFAP demonstrated enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared to control animals (Fig. 7 [C1-C4]).

In CA3 subfield of the hippocampus, the thickness and neuronal density of stratum pyramidale appeared reduced in all three treated groups compared to control animals (Fig. 8). The degenerating neurons were conspicuous in the stratum pyramidale of animals treated with DEET alone (Fig. 8 [A1-A2]). The MAP-2 staining of adjacent sections demonstrated only a slightly reduced density of MAP-2 positive dendrites in animals belonging to all three treated groups compared to control animals (data not shown). The GFAP immunostaining of neighboring sections showed enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared to control animals (Fig. 8 [C1-C4]).

Extent of neuron loss, reductions in MAP-2 immunoreactivity, and up-regulation of GFAP immunoreactivity in the hippocampal formation

Quantification of surviving and dying neurons per mm^2 area of granule cell layer of the dentate gyrus and pyramidal cell layer of subfields CA1 and CA3 (Fig. 9) demonstrated the following. In dentate granule cell layer, animals treated with DEET, permethrin or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($p < 0.01$; Fig. 9). Further, animals treated with either DEET or permethrin exhibited a significant decrease in surviving neurons, in comparison to animals treated with combined DEET and permethrin ($p < 0.01$; Fig. 9). Analysis of dying

neurons showed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($p < 0.05$; Fig. 9). In CA1 subfield, only animals treated with either DEET or permethrin exhibited a significant decrease in the number of surviving neurons compared to control animals ($p < 0.05$; Fig. 9). Analysis of dying neurons also showed the same trend. In CA3 subfield, animals treated with DEET, permethrin or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($p < 0.01$; Fig. 9). Analysis of dying neurons revealed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($p < 0.01$). Thus, a significant reduction in surviving neurons occurs in the dentate gyrus and CA3 subfield of the hippocampal formation following sub-chronic dermal application of DEET and permethrin, alone or in combination; however, the overall neuron loss in the dentate gyrus is significantly greater when DEET and permethrin are applied separately. In CA1 subfield of the hippocampus, a significant decrease in the number of surviving neurons occurs with exposure to DEET or permethrin alone but not with exposure to both DEET and permethrin.

Quantification of the area of MAP-2 immunoreactive elements per unit area of the CA1 stratum radiatum showed that there were fewer MAP-2 positive structures in the CA1 subfield of all treated groups (Fig. 10 [A]). The MAP-2 immunoreactive structures exhibited 28% reduction with exposure to DEET alone ($p < 0.05$), and 16% reduction with exposure to permethrin alone or exposure to both DEET and permethrin ($p < 0.05$). The measurement of GFAP immunoreactive structures per unit area of different regions of the hippocampal formation demonstrated up-regulation in GFAP positive elements in all treatment groups (Fig. 10 [B]). In dentate gyrus, the increase was 77% with DEET exposure ($p < 0.05$), 65% with permethrin exposure ($p < 0.05$), and 24% with exposure to both DEET and permethrin ($p < 0.05$). In the CA1 subfield, the increase in different treatment groups varied from 81-91% ($p < 0.05$). In the CA3 subfield, the increase in GFAP immunoreactivity varied from 60-93% ($p < 0.05$).

Alterations in the cytoarchitecture of the cerebellum

In the cerebellum, the most conspicuous damage following exposure to DEET and permethrin, alone or in combination, was in the Purkinje cell layer. A large number of degenerating neurons were observed in animals treated with either DEET or permethrin compared to control animals (Fig. 11 [A1-A3]). In animals treated with combined DEET and permethrin, dying neurons were infrequent. However, the Purkinje cell density per length of Purkinje cell layer appeared reduced in comparison to control animals (Fig. 11 [A4]). Wide areas of the Purkinje cell layer lacking Purkinje neurons were frequently encountered in animals treated with both DEET and permethrin (Fig. 11 [A4]). Both thickness and cell packing density in granule cell layer appeared comparable between control animals and animals belonging to three treated groups. The GFAP immunostaining of neighboring sections showed a significantly enhanced GFAP immunoreactivity in the cerebellar white matter of animals belonging to the three treatment groups compared to control animals (Fig. 11 [B1-B4]). However, the maximal enhancement in GFAP immunoreactivity was observed in animals treated with both DEET and permethrin (Fig. 11 [B4]).

Extent of Purkinje neuron loss, and up-regulation of GFAP immunoreactivity in the cerebellum

Quantitative analysis of Purkinje cells showed that animals treated with DEET and permethrin, alone or in combination, exhibited a significant decrease in surviving neurons, in comparison to control animals (76-83% in lobule 2 of the cerebellar vermis, and 36-58% in crus 2 ansiform lobule of the cerebellar hemisphere, $p < 0.001$; Fig. 12). However, the degree of neuron loss in the cerebellar hemisphere with combined exposure to DEET and permethrin was greater than exposure to DEET or permethrin alone ($p < 0.05$; Fig. 12). Analysis of dying neurons in the cerebellar vermis revealed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($p < 0.001$; Fig. 12). However, in the cerebellar hemisphere, all treated groups exhibited a significant number of dying neurons ($p < 0.05$). Thus, as in the cerebral cortex and the hippocampal formation, a significant neuronal cell death occurs in the Purkinje cell layer of the cerebellum following sub-chronic dermal application of DEET and

permethrin, alone or in combination; however, the overall neuron loss in the cerebellar hemisphere is significantly greater with combined application of DEET and permethrin, compared to exposure to DEET or permethrin alone. The measurement of GFAP immunoreactive structures per unit area of the central white matter of the cerebellum demonstrated significant up-regulation in GFAP positive elements in all treatment groups (53-60% increase with exposure to DEET or permethrin ($p < 0.05$, Fig. 12 [C]), and 106% increase with exposure to both DEET and permethrin ($p < 0.01$, Fig. 12 [C]).

DISCUSSION

The present study was designed to investigate the effects of daily dermal application of DEET and permethrin, alone or in combination, for 60 days on histopathological changes in the brain of male rats. The route of exposure and dose levels of test compounds were chosen to closely reflect those present during the Persian Gulf War [1]. The test-compounds were applied dermally at a dose, which was approximately equivalent to the exposure that may have occurred to army personnel during the Gulf War [18, Dr. W. McCain, Department of Defense, Personal communication]. Our data suggest that exposure to DEET and permethrin, alone or in combination, for 60 days causes the following. (1) A diffuse neuronal cell death in the motor cortex, the different subfields of the hippocampal formation and the Purkinje cell layer of the cerebellum. (2) A significant reduction in MAP-2 positive immunoreactive structures associated with atypical expression of MAP-2 in dendrites of surviving neurons within the cerebral cortex and the hippocampus; the expression of MAP-2 within apical dendrites of pyramidal neurons of the cortex and the CA1 subfield was characterized by a beaded, disrupted, or wavy appearance. (3) A significant up-regulation of GFAP positive structures. This was exemplified by GFAP expression in soma of astrocytes and hypertrophy of astrocytic processes emanating from the soma.

To determine the overall extent of neuron loss, we quantified the density of surviving (healthy) and dying neurons in layers III and V of the motor cerebral cortex, granule cells of the dentate gyrus, pyramidal neurons of hippocampal CA1 and CA3 subfields, and Purkinje cells of the cerebellum. The neuronal cell

death was evident in all three treated groups by a significant decrease in the density of surviving neurons. In animals treated with DEET or permethrin alone, the occurrence of neuronal cell death was also confirmed by the presence of a significant number of dying (eosinophilic) neurons after the 60 days exposure regimen. However, in animals treated with both DEET and permethrin, the number of dying neurons after the same exposure regimen was significantly less than animals treated with DEET or permethrin alone. A lack of significant number of dying neurons but a clear reduction in the number of surviving neurons (in comparison to control animals) in animals exposed to both DEET and permethrin suggests that, in animals receiving combined DEET and permethrin, neuronal cell death occurs earlier than animals receiving either DEET or permethrin alone. However, analysis of dying neurons at multiple time-points during the exposure period employed in this study are necessary to clearly address the above issue. Further, the extent of reductions in surviving neurons within some regions of the brain varied between the three treatment groups. The layer III and V of the motor region of the cerebral cortex exhibited similar level of reductions (43-57%) following exposure to DEET and permethrin, alone or in combination. The dentate gyrus of the hippocampal formation demonstrated a significantly greater level of reduction with exposure to DEET or permethrin alone, in comparison to combined DEET and permethrin exposure (54% with DEET alone, 54% with permethrin alone, and 29% with combined DEET and permethrin); however, the CA1 and CA3 subfields of the hippocampus exhibited statistically similar level of reductions (CA1, 33-51% reduction; CA3, 38-53% reduction) following exposure to DEET and permethrin, alone or in combination. The Purkinje cells of the cerebellar vermis exhibited similar level of reductions with all three exposures (76-83% reduction). However, the Purkinje cells of the cerebellar hemisphere demonstrated a significantly greater reduction with combined exposure to DEET and permethrin (58% decrease) than exposure to DEET or permethrin alone (36-43% decrease). Thus, in the motor cerebral cortex, the hippocampal subfields CA1 and CA3, and the Purkinje cell layer of the cerebellar vermis, exposure to DEET and permethrin, alone or in combination, causes similar level of neuronal cell death. In contrast, in the dentate gyrus, exposure to DEET alone causes greater neuronal cell death than exposure to both DEET and permethrin. And, in the Purkinje cell layer of the cerebellar

hemisphere, exposure to combined DEET and permethrin causes more damage than DEET or permethrin alone.

A greater level of neuron loss in the dentate gyrus following exposure to DEET alone compared to exposure to a combination of DEET and permethrin suggests that the extent of DEET-induced neuronal loss in some regions of the brain wanes significantly when both DEET and permethrin were applied together. The reduced neuronal loss with exposure to both DEET and permethrin likely reflects a decrease in effective concentration of chemicals at the neurotoxicity target, as concurrent exposure to chemicals can decrease their absorption [18,24]. This may also suggest that there is some protective effect with concurrent exposure to DEET and permethrin. Nevertheless, animals treated with both DEET and permethrin exhibited maximal cytoarchitectural alterations in the expression of MAP-2 within the motor cerebral cortex and the CA1 subfield of the hippocampus though the overall reductions in MAP-2 immunoreactivity was mostly comparable in all treated groups. The up-regulation of GFAP immunoreactivity was comparable between the three treatment groups in the CA3 subfield of the hippocampus. In the motor cortex and the dentate gyrus, the up-regulation was greater with exposure to DEET or permethrin alone compared to combined exposure; whereas, in the cerebellum, the up-regulation was greater with combined exposure than with exposure to DEET or permethrin alone. The above pattern of cytoskeletal alterations suggest that degenerative changes induced by co-exposure to DEET and permethrin are significant and detrimental to the normal functioning of the central nervous system, despite a slight reduction in the extent of overall neuronal cell loss.

In our previous studies in hens using sub-cutaneous route of exposure at relatively higher doses, we demonstrated that co-exposure to DEET and permethrin results in an enhanced level of toxicity than exposure to each chemical alone [2]. Our recent behavioral studies in rats showed that exposure to DEET or permethrin or both DEET and permethrin for 60 days using the same dose level used in the current study, leads to significant deficits in sensorimotor functions [1]. In addition, our previous study demonstrated subtle changes in the blood brain barrier following exposure to either DEET or both DEET and permethrin, and suggested that additional approaches such as histopathological evaluations may

provide definitive proof of the changes in the CNS occurring as a consequence of DEET treatment alone or a combination with permethrin. Our present data provide clear histopathological evidence that sub-chronic exposure to DEET and permethrin, alone or in combination, leads to a significant neuronal cell death and cytoskeletal abnormalities in surviving neurons that could compromise functions of the brain. The neurotoxic effects of DEET may be augmented, by both its increased localization into the CNS because of its lipophilicity, and because of decrease in the transport of otherwise critical molecules. Severe signs of CNS toxicity due to DEET and permethrin are apparent only at high doses, e.g., DEET induced signs of CNS depression, death and protracted seizure activity was observed at several dose levels in rats [39]. Similar complications have been observed in DEET poisoning in humans [21,26]. Symptoms such as daytime sleepiness and impaired cognitive functions have been shown to result from heavy DEET exposure, whereas gait balance and dexterity were moderately affected [21]. Additionally, lethargy has been noted as a prominent feature in severe acute DEET intoxication [33,35]. A relatively recent study found a decrease in motor activity in male and female rats after a single dose of DEET treatment [39]. Permethrin induced behavioral changes have also been documented in animals [16]. Permethrin-induced neurotoxic changes are characterized by aggressive sparring, increased sensitivity to external stimuli and fine tremors that progresses to whole-body tremors and prostration [4,37,38]. McDaniel and Moser [22] reported a decrease in grip strength and induced head and forelimb shaking. Additionally, decreased operant response rate, and a decrease in turning-wheel activity have been observed [6,14]. Studies by Crofton and Reiter [9] have shown a decrease in locomotor activity in rats exposed to permethrin.

The cytoarchitecture of the CNS is maintained by a complex cellular milieu that involves neurons and a variety of cells of glial origin. In order for the CNS to function properly and to respond to external stimuli, it is absolutely required that a proper communication is maintained within these cells. A major determinant of neuronal morphology is the cytoskeleton. Different components of cytoskeleton within the neurons and astrocytes provide forces to maintain the appropriate cellular structure, e.g., neuronal dendrites and axons are maintained in stable conditions by the force provided by the elements of

cytoskeleton [13]. Such interactions are essential for proper synapse formation. The components of cytoskeleton are microfilament, intermediate filaments and microtubules. An important neuronal component, MAP-2 is enriched in dendrites and cell bodies [36], in which it stabilizes the polymerized tubulin. Abnormal regulation of expression of MAP-2 causes suppression of neurite outgrowth and reduction in the number of neurites in cultured neurons [7]. Similarly, aberrant intermediate filament proteins have been linked to diseases of neurodegeneration [11]. Our data clearly show both decrease and abnormalities in MAP-2 expression following treatment with DEET or permethrin or both DEET and permethrin in the brain, particularly the motor cerebral cortex and the CA1 subfield of the hippocampal formation. A decreased expression and beaded appearance of MAP-2 in dendrites would lead to destabilization of dendrites and can result in abnormal functioning of neurons particularly loss of synapses due to resorption of post-synaptic specializations such as dendritic spines. Such aberrant dendritic organization and the consequently altered connectivity in the cerebral cortex and the hippocampal formation could respectively have profound adverse influence on motor function and learning and memory.

A major component of astrocytic intermediate filament, GFAP is up-regulated in response to reactive gliosis as a consequence of a variety of insults, such as exposure to neurotoxic chemicals, trauma, and neurodegenerative diseases that affect the CNS [12]. The precise function of GFAP is not well understood, but it is believed to play an important role in the long-term maintenance of brain cytoarchitecture [19], proper functioning of the blood brain barrier [25], and modulation of neuronal functions [31]. An increased expression of GFAP in the soma and processes of astrocytes in various brain regions exhibiting neuronal cell death indicates that neurodegenerative changes induced by exposure to DEET and permethrin either alone or in combination are quite robust and lead to a significant hypertrophy of astrocytes. This is because hypertrophied astrocytes (or reactive astrocytes) represent transformed resting astrocytes with increased GFAP accumulation, and this transformation occurs as a consequence of injury to the brain. The accumulation of reactive astrocytes can lead to increased generation of toxic mediators that may cause further pathological damages in the brain [27].

Conclusions

Most of the earlier studies on the neurotoxic effects of DEET or permethrin used routes of exposure that are not directly germane to the contact exposure, as is believed to have occurred during the Gulf War. The results of this study, however, clearly suggest that sub-chronic dermal application of these chemicals leads to a diffuse neuronal cell death and significant neuronal cytoskeletal abnormalities in the motor cerebral cortex, the hippocampal formation, and the cerebellum. Taken together, these alterations can lead to many physiological and behavioral abnormalities particularly motor deficits and learning and memory dysfunction. The above alterations are likely the contributory factors for neurobehavioral abnormalities observed earlier in adult rats following exposure to DEET and permethrin, alone or in combination. Thus, it is likely that sub-chronic exposure to DEET and permethrin experienced by service personnel during the Persian Gulf War have played an important role in the development of illnesses in some veterans after the Gulf War.

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FIGURE LEGENDS

Figure 1 – Effect of daily administration of DEET (40mg/kg/d, dermal) and permethrin (0.1340 mg/kg/d, dermal) on body weight of rats. Animals were assessed for body weight each week. The percentage of initial body weight for control (Mean \pm SEM) is 118 \pm 6.7 (week 1), 122 \pm 7.1 (week 2), 135 \pm 5.8 (week 3), 140 \pm 6.1 (week 4), 145 \pm 6.5 (week 5), 155 \pm 8.5 (week 6), 157 \pm 10.7 (week 7), 164 \pm 9.4 (week 8). Analysis with one-way ANOVA with Student Newman-Keuls multiple comparisons test revealed no significant differences between groups at all time-points during the exposure regimen ($p > 0.1$).

Figure 2 - Alterations in the superficial layers (layers I-III) of the motor cortex following daily dermal application of DEET and permethrin. A1-A4, H&E staining; B1-B4, MAP-2 immunostaining; C1-C4, GFAP immunostaining. A1, B1, C1 are examples from a control rat. A2, B2, C2 are examples from a rat treated with DEET. A3, B3, C3 are examples from a rat treated with permethrin whereas A4, B4, C4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in rats treated with either DEET or permethrin (arrows in A2 and A3). Whereas in the rat treated with both DEET and permethrin, vacant areas devoid of neurons (A4) and wavy appearance of dendrites (arrows in B4) are conspicuous. Note that, in all three treated groups, the overall MAP-2 immunoreactivity is significantly reduced (B2-B4) and GFAP immunoreactivity of astrocytes is significantly enhanced (arrowheads in C2-C4), in comparison to the control group (B1, C1). Scale bar, 100 μ m.

Figure 3- Changes in the deeper layers (layers IV-V) of the motor cortex following daily application of DEET and permethrin. A1-A4, H&E staining; B1-B4, MAP-2 immunostaining; C1-C4, GFAP immunostaining. A1, B1, C1 are examples from a control rat. A2, B2, C2 are examples from a rat treated with DEET. A3, B3, C3 are examples from a rat treated with permethrin whereas A4, B4, C4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating pyramidal

neurons are clearly visible in layer V of the cortex in rats treated with either DEET or permethrin (arrows in A2 and A3). In the rat treated with both DEET and permethrin, vacant areas devoid of neurons (A4) and wavy appearance of dendrites (arrows in B4) are conspicuous. Note that, in all three treated groups, the overall MAP-2 immunoreactivity is significantly reduced (B2-B4) and GFAP immunoreactivity of astrocytes is significantly enhanced (arrows in C2-C4), in comparison to the control group (B1, C1). Scale bar, 100 μ m.

Figure 4 – Histograms show the density of surviving (A) and dying (B) neurons/mm² area of layers III and V of the motor cortex. Values represent means and standard errors (n = 5 per group). Analyses with one-way ANOVA shows significant differences for surviving neurons between groups (p < 0.01 in layer III, p < 0.001 in layer V). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further revealed that animals treated with DEET or permethrin alone exhibit a significant decrease in the number of surviving neurons, in comparison to control animals (layer III, p < 0.05; layer V, p < 0.01). Analysis of dying neurons revealed that, in layer III of the motor cortex, animals treated with DEET exhibit a significant increase in the number of dying neurons, in comparison to control animals, animals treated with permethrin, and animals treated with both DEET and permethrin (p < 0.01). In layer V of the motor cortex, animals treated with either DEET or permethrin exhibit a significant increase in dying neurons compared to control animals (p < 0.001 and p < 0.01 respectively). Further, exposure to DEET alone results in a significantly increased number of dying neurons than exposure to permethrin alone (p < 0.05) and exposure to both DEET and permethrin (p < 0.001).

Figure 5 – Histograms in A show the area of MAP-2 immunoreactive elements in mm² per unit area (0.044 mm²) of layers III and V of the motor cortex. Values represent means and standard errors (n = 5 per group). In layer III of the cortex, the MAP-2 immunoreactive structures exhibited 27-28% reduction with exposure to DEET or permethrin alone (p < 0.05), and 15% reduction with exposure to both DEET and permethrin (p > 0.05). Whereas, in layer V of the motor cortex, the MAP-2 immunopositive

structures showed 52% reduction with exposure to DEET alone ($p < 0.01$), 35% reduction with exposure to permethrin alone ($p < 0.05$), and 49% reduction with exposure to both DEET and permethrin ($p < 0.01$). Histograms in B show the area of GFAP immunoreactive elements (in mm^2) per unit area (0.176 mm^2) of layer V of the motor cortex. Values represent means and standard errors ($n = 5$ per group). Note a significant up-regulation in GFAP positive elements ($p < 0.05$) with exposure to either DEET (74% increase) or permethrin (63% increase). However, with combined exposure to DEET and permethrin, there was only a 10% increase in GFAP immunoreactivity ($p > 0.05$).

Figure 6 – Alterations changes in the dentate gyrus following daily application of DEET and permethrin. A1-A4, H&E staining; B1-B4, GFAP immunostaining. A1, B1 are examples from a control rat. A2, B2 are examples from a rat treated with DEET. A3, B3 are examples from a rat treated with permethrin whereas A4, B4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in the dentate granule cell layer (GCL) and the dentate hilus (DH) of rats treated with either DEET or permethrin (arrows in A2 and A3). In the rat treated with both DEET and permethrin (A4), both thickness and cell packing density of granule cell layer are reduced compared to the control rat. Note that GFAP immunoreactivity is up regulated in all three treated groups (B2, B3, B4). ML, molecular layer. Scale bar, 100 μm .

Figure 7 - Alterations in the CA1 subfield of the hippocampus following daily dermal application of DEET and permethrin. A1-A4, H&E staining; B1-B4, MAP-2 immunostaining; C1-C4, GFAP immunostaining. A1, B1, C1 are examples from a control rat. A2, B2, C2 are examples from a rat treated with DEET. A3, B3, C3 are examples from a rat treated with permethrin whereas A4, B4, C4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating pyramidal neurons are clearly visible in the stratum pyramidale (SP) of rats treated with either DEET or permethrin (arrows in A2 and A3). In the rat treated with both DEET and permethrin, both thickness and cell packing density of CA1 cell layer are reduced compared to the control rat (A4) and dendrites in stratum radiatum

have wavy appearance (B4). Note that, in all three treated groups, the overall density of MAP-2 immunoreactive elements is significantly reduced (B2-B4) and the pattern of MAP-2 expression in dendrites is altered, in comparison to the control group (B1). Further, in all treatment groups (C2-C4), GFAP immunoreactive astrocytes are significantly increased in both stratum oriens (SO) and stratum radiatum (SR). Scale bar, 100 μ m.

Figure 8 - Changes in the CA3 subfield of the hippocampus following daily application of DEET and permethrin. A1-A4, H&E staining; B1-B4, GFAP immunostaining. A1, B1 are examples from a control rat. A2, B2 are examples from a rat treated with DEET. A3, B3 are examples from a rat treated with permethrin whereas A4, B4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in the stratum pyramidale (SP) of CA3 subfield of the rat treated with DEET alone (arrows in A2). In rats treated with permethrin alone (A3) and both DEET and permethrin (A4), the thickness and cell packing density of CA3 cell layer are reduced compared to the control rat. Note that GFAP immunoreactivity is up regulated in all three treated groups (B2, B3, B4). SO, stratum oriens; Scale bar, 100 μ m.

Figure 9 – Histograms show the density of surviving (A) and dying (B) neurons per mm² area of different cell layers of the hippocampal formation. Values represent means and standard errors (n = 5 per group). Analyses with one-way ANOVA revealed significant differences between groups for both surviving neurons (dentate gyrus, p < 0.001; CA1 subfield, p < 0.05; CA3 subfield, p < 0.001) and dying neurons (dentate gyrus, CA1 and CA3 subfields, p < 0.01). The post-hoc analysis with Student's Newman-Keuls multiple comparisons test further revealed the following. In dentate granule cell layer, animals treated with DEET, permethrin or a combination of DEET and permethrin exhibit a significant decrease in surviving neurons, in comparison to control animals (p < 0.01). Further, animals treated with either DEET or permethrin exhibit a significant decrease in surviving neurons, in comparison to animals treated with both DEET and permethrin (p < 0.01). Analysis of dying neurons (B) shows that animals

treated with either DEET or permethrin exhibit a significant number of dying neurons compared to control animals ($p < 0.05$); the number of dying neurons with exposure to DEET alone is greater than with exposure to both DEET and permethrin ($p < 0.05$). In CA1 subfield, only animals treated with either DEET or permethrin exhibit a significant decrease in the number of surviving neurons compared to controls ($p < 0.05$). Analysis of dying neurons shows that animals treated with either DEET or permethrin exhibit a greater number of dying neurons than both controls ($p < 0.01$) and animals treated with both DEET and permethrin ($p < 0.05$). In CA3 subfield, animals treated with DEET, permethrin or a combination of DEET and permethrin exhibit a significant decrease in surviving neurons, in comparison to controls ($p < 0.01$). Analysis of dying neurons reveals that animals treated with either DEET or permethrin exhibit a greater number of dying neurons compared to both controls ($p < 0.01$) and animals treated with both DEET and permethrin ($p < 0.05$).

Figure 10 - Histograms in A show the area of MAP-2 immunoreactive elements (in mm^2) per unit area (0.044 mm^2) of CA1 stratum radiatum of the hippocampus. Values represent means and standard errors ($n = 5$ per group). Note that there are fewer MAP-2 positive structures in the CA1 subfield of all treated groups. The MAP-2 immunoreactive structures exhibited 28% reduction with exposure to DEET alone ($p < 0.05$), and 16% reduction with exposure to permethrin alone or exposure to both DEET and permethrin ($p < 0.05$). Histograms in B show the area of GFAP immunoreactive elements in mm^2 per unit area (0.176 mm^2) of different regions of the hippocampus. Values represent means and standard errors ($n = 5$ per group). Note the up-regulation in GFAP positive elements within all regions of the hippocampus in all treatment groups. In dentate gyrus, the increase was 77% with DEET exposure ($p < 0.05$), 65% with permethrin exposure ($p < 0.05$), and 24% with exposure to both DEET and permethrin ($p < 0.05$). In the CA1 subfield, the increase in different treatment groups varied from 81-91% ($p < 0.05$). In the CA3 subfield, the increase in GFAP immunoreactivity varied from 60-93% ($p < 0.05$).

Figure 11 – Alterations in the cerebellum following daily application of DEET and permethrin. A1-A4, H&E staining of the cerebellar cortex; B1-B4, GFAP immunostaining of the cerebellar white matter. A1, B1 are examples from a control rat. A2, B2 are examples from a rat treated with DEET. A3, B3 are examples from a rat treated with permethrin whereas A4, B4 are examples from a rat treated with both DEET and permethrin. A large number of degenerating Purkinje neurons are clearly visible in the Purkinje cell layer of rats treated with either DEET or permethrin (arrows in A2, A3). Arrows in A1-A4 point to surviving neurons. In rats treated with both DEET and permethrin (A4), a large area of Purkinje cell layer is devoid of Purkinje neurons (bracketed area A4). Note that GFAP immunoreactivity in the white matter of the cerebellum is significantly up regulated in all three treated groups (B2, B3, B4) with maximal up-regulation in the group treated with both DEET and permethrin. GCL, granule cell layer; ML, molecular layer. Scale bar, 100 μ m.

Figure 12 - Histograms in A and B show the density of surviving (A) and dying (B) neurons per mm^2 area of the Purkinje cell layer in lobule 2 of the cerebellar vermis and crus 2 ansiform lobule of the cerebellar hemisphere. Values represent means and standard errors ($n = 5$ per group). Note that animals treated with DEET and permethrin, alone or in combination, exhibit a significant decrease in surviving neurons, in comparison to control animals (76-83% in lobule 2 of the cerebellar vermis, and 36-58% in crus 2 ansiform lobule of the cerebellar hemisphere, $p < 0.001$). And, the degree of neuron loss in the cerebellar hemisphere with combined exposure to DEET and permethrin is greater than exposure to DEET or permethrin alone ($p < 0.05$). Also, Animals treated with either DEET or permethrin exhibit a significant number of dying neurons in the cerebellar vermis ($p < 0.001$; B). In the cerebellar hemisphere, all treated groups exhibit a significant number of dying neurons ($p < 0.05$). Histograms in C show GFAP immunoreactive elements in mm^2 per unit area (0.176 mm^2) of the central white matter of the cerebellum. Note that a significant up-regulation in GFAP positive elements occurs in all treatment groups (53-60% increase with exposure to DEET or permethrin ($p < 0.05$), and 106% increase with exposure to both DEET and permethrin ($p < 0.01$).

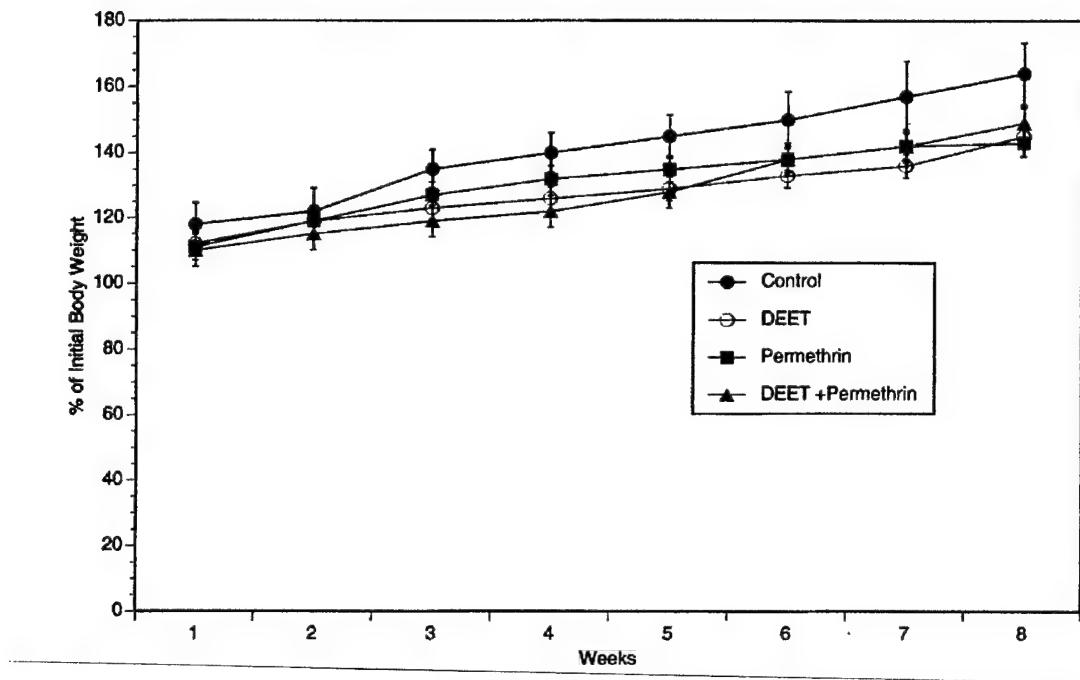


FIGURE 1

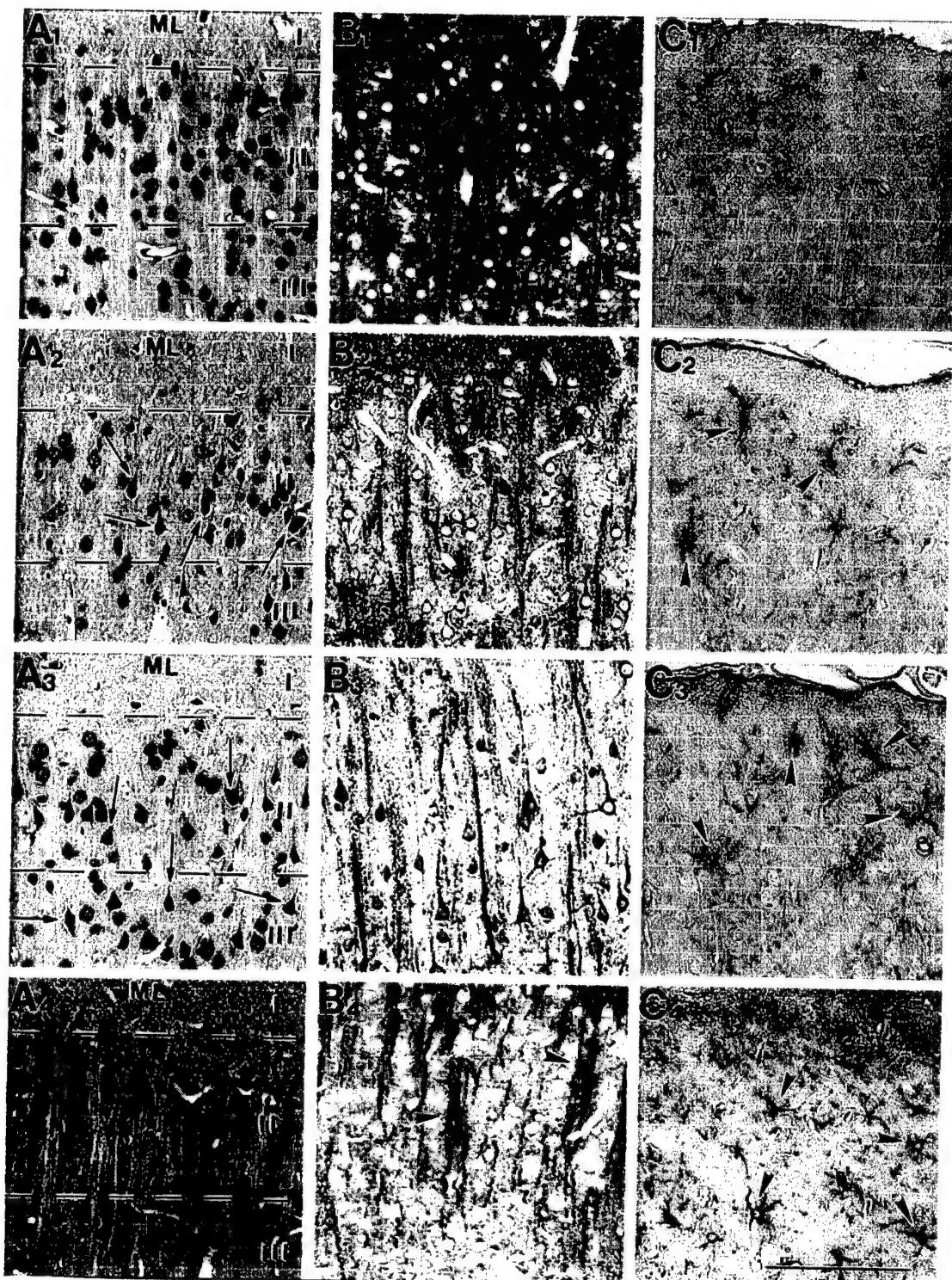


FIGURE 2

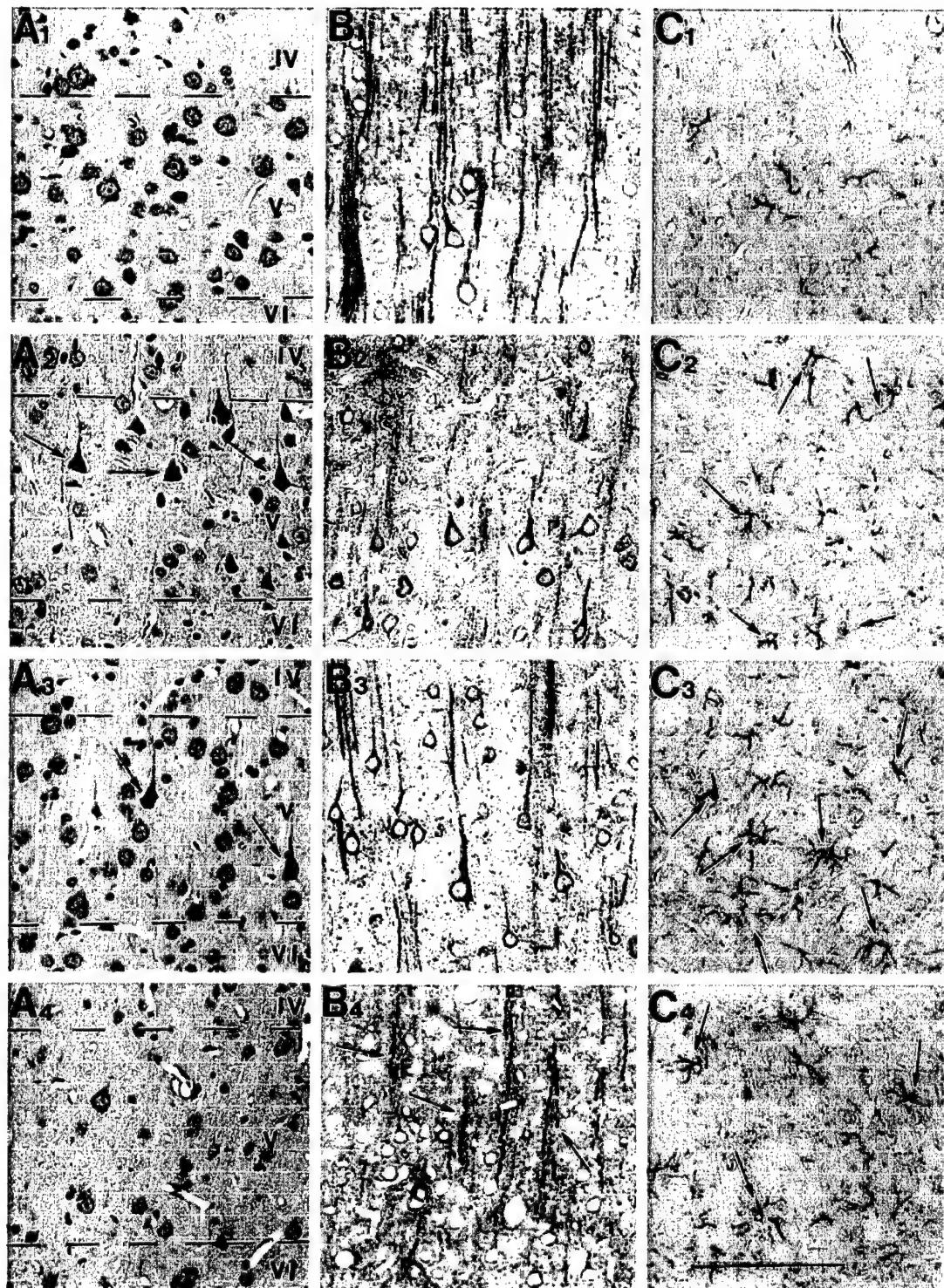


FIGURE 3

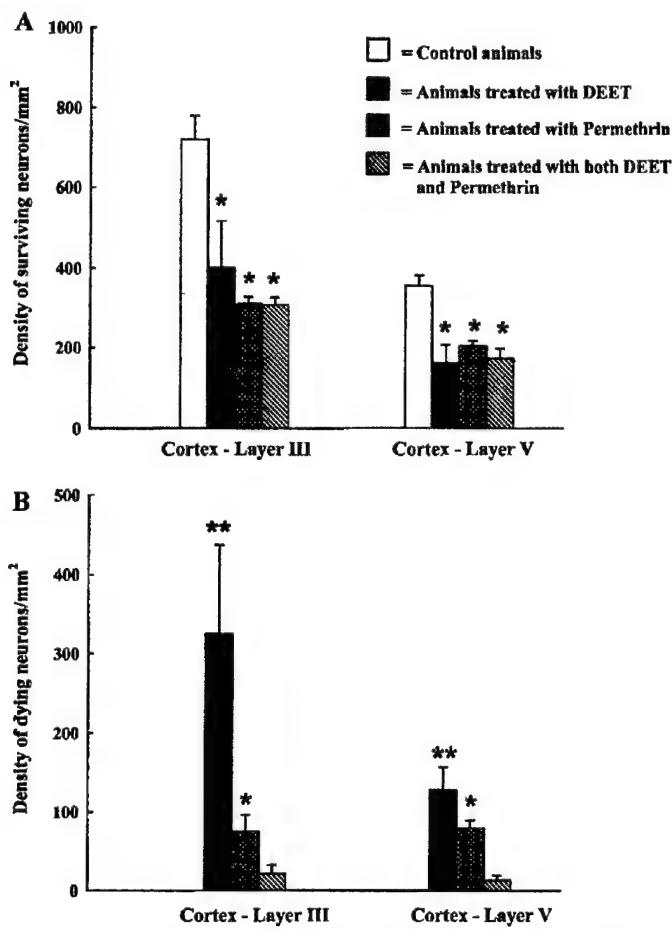


FIGURE 4

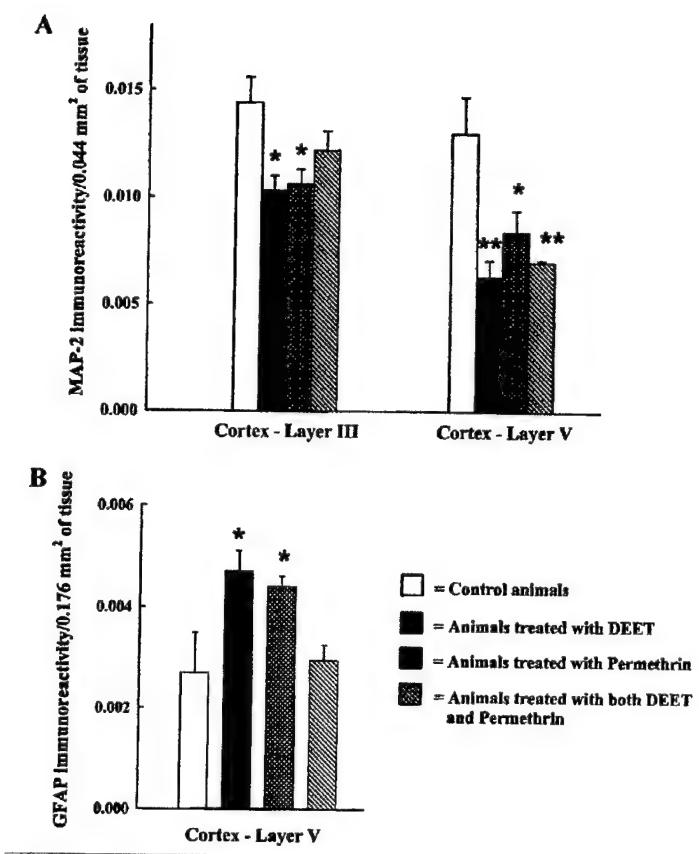


FIGURE 5

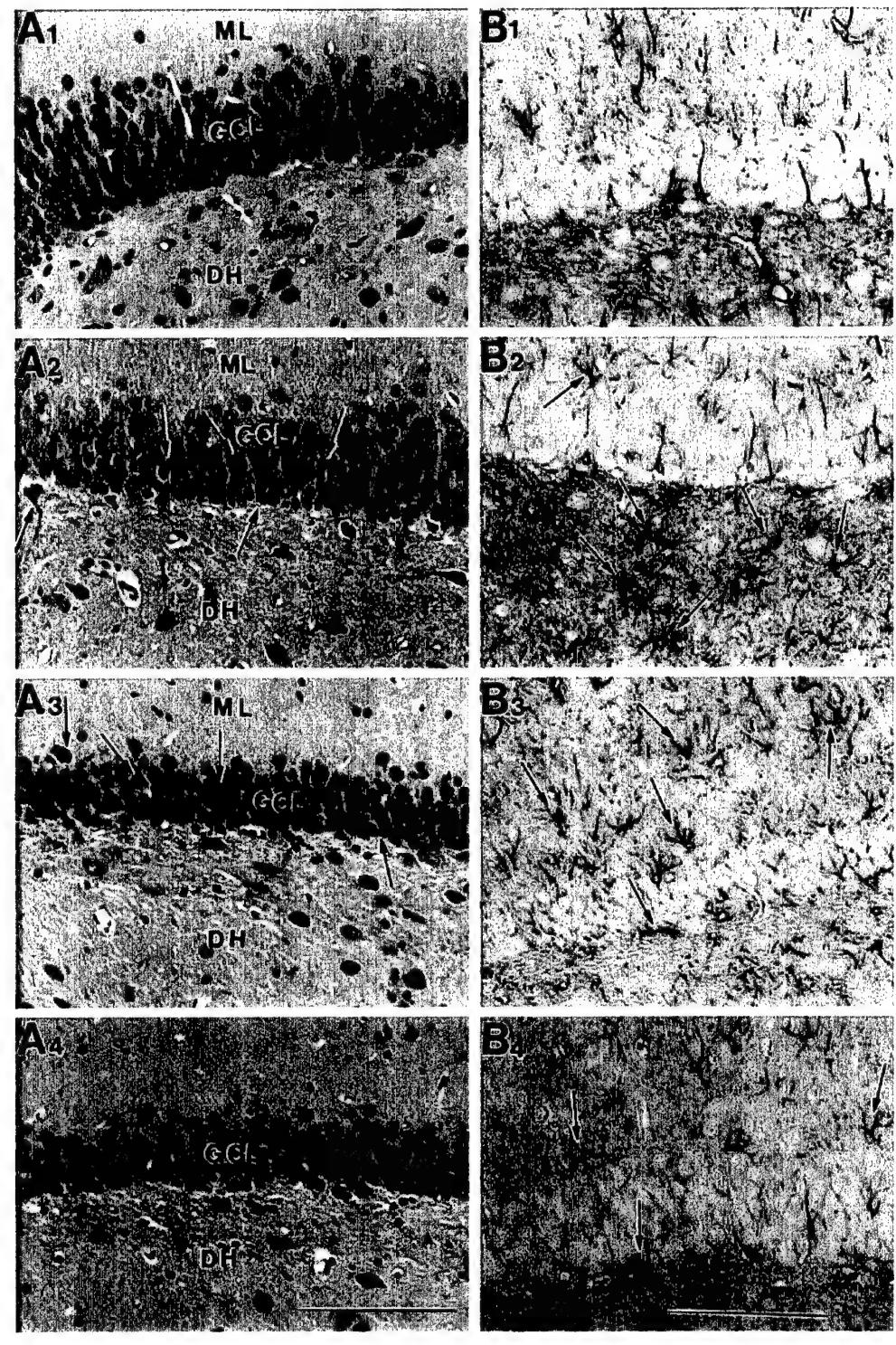


FIGURE 6

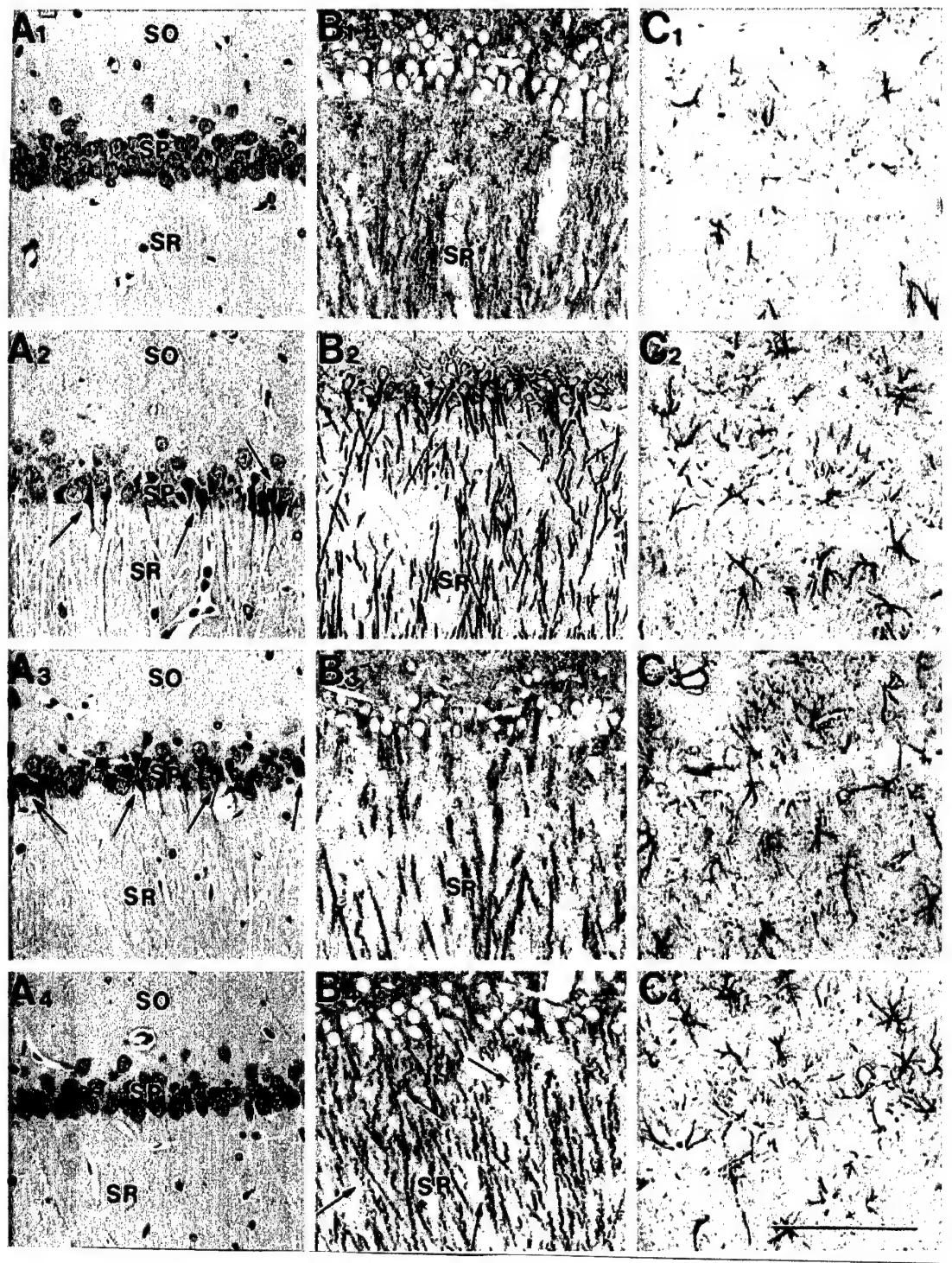


FIGURE 7

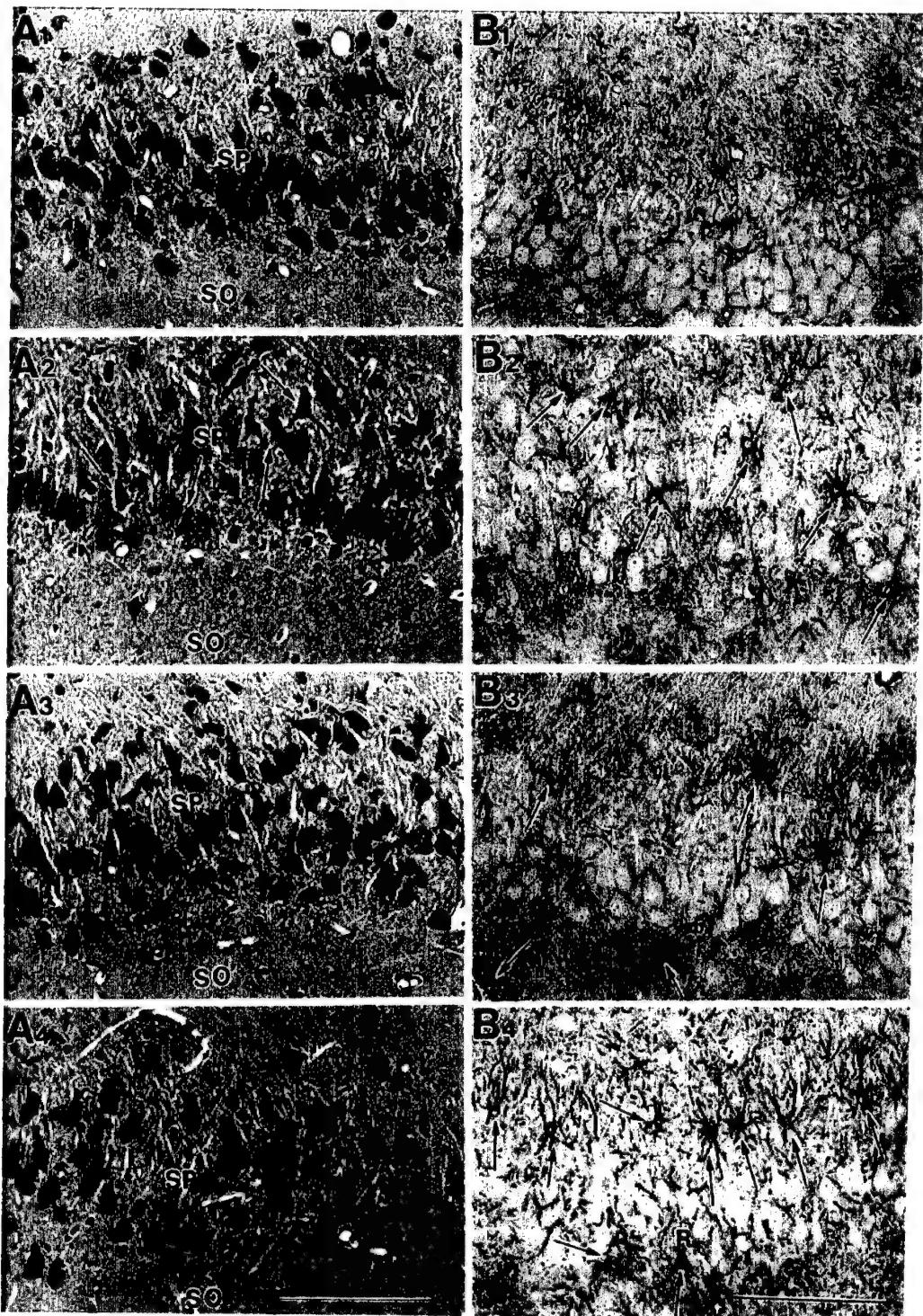


FIGURE 8

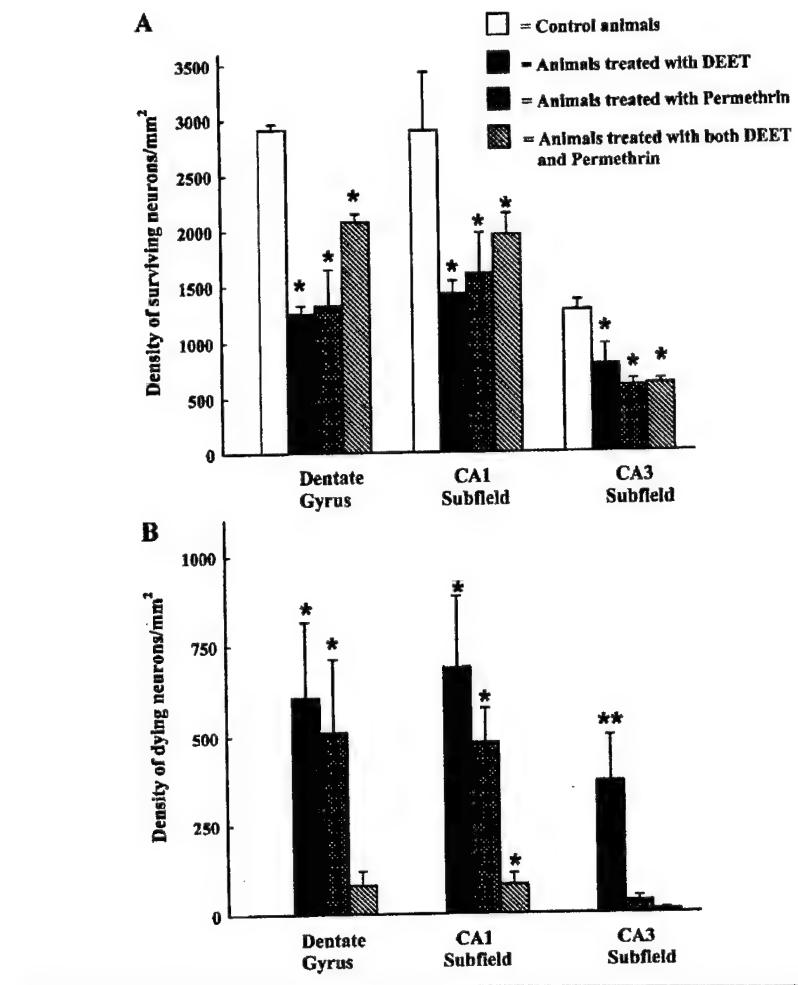


FIGURE 9

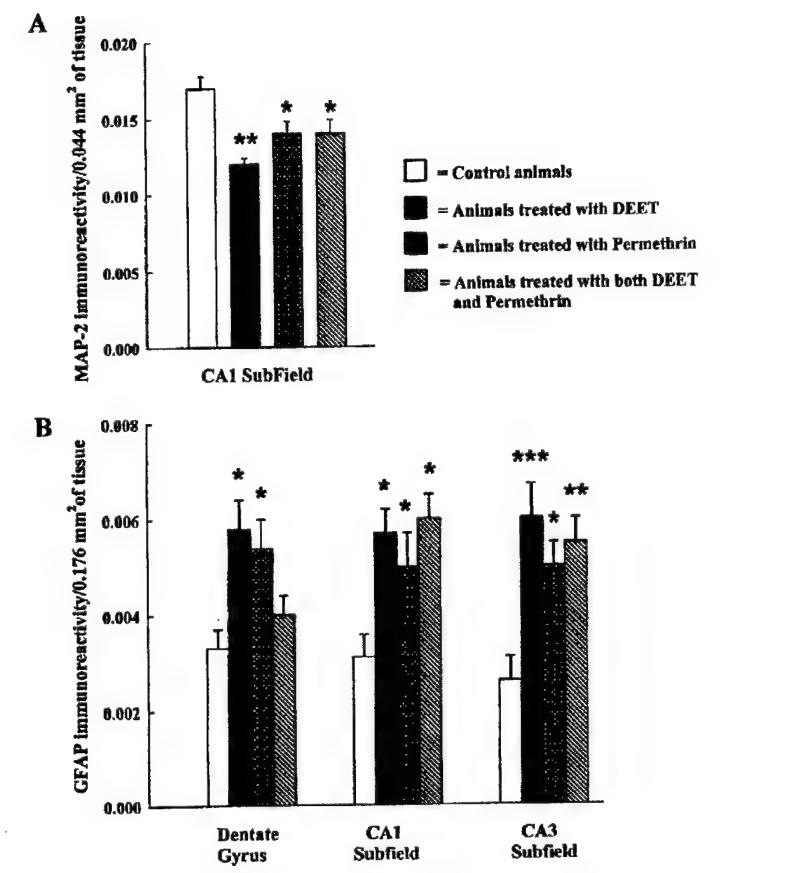


FIGURE 10

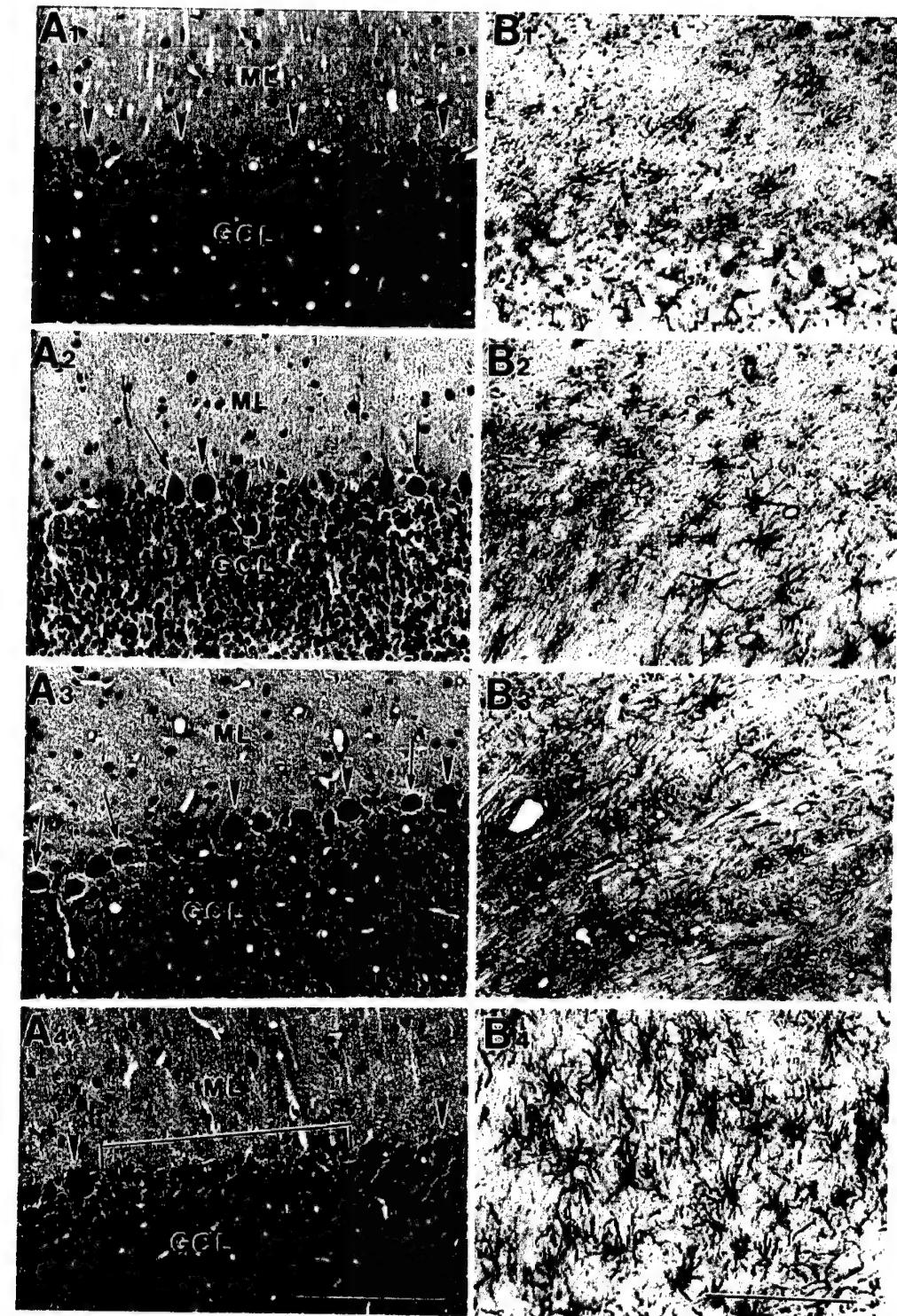


FIGURE 11

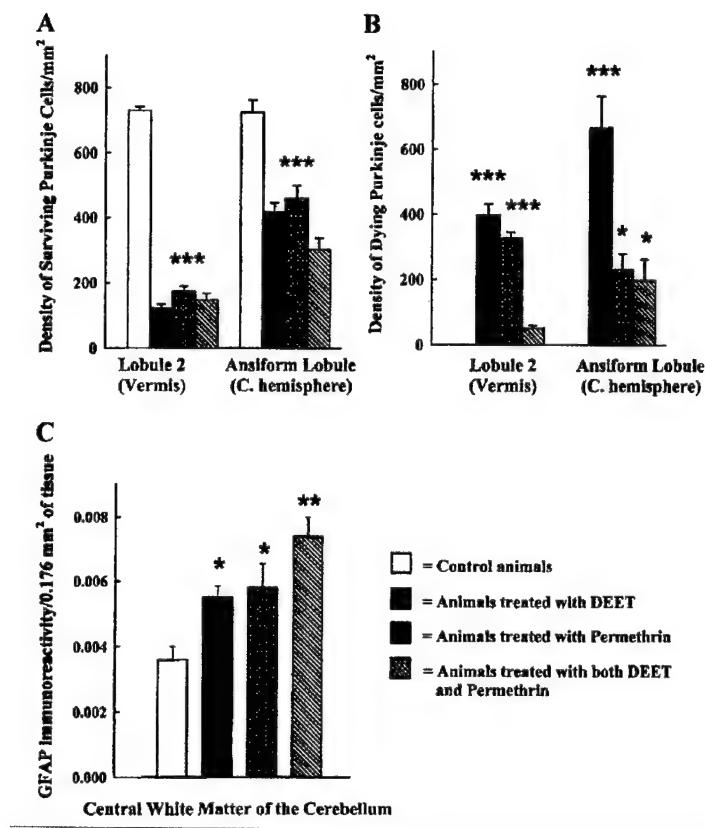


FIGURE 12

2. Effect of Dermal Application of DEET and Permethrin, Alone and In Combination On the Release of Rat Brain Mitochondrial Cytochrome c

Abstract

The release of cytochrome *c* from the mitochondrial intermembrane space can induce apoptosis. The Levels of mitochondrial cytochrome *c* in rat brain following a single dermal dose of 400 mg/kg of DEET, and a 1.3 mg/kg of permethrin, alone or in combination were determined. Rats were sacrificed at time interval of 0.5, 1, 2, 4, 8, 16, 24, 48, or 72 h after dosing. Brain mitochondria were isolated and the levels of cytochrome *c* measured using reversed phase-high performance liquid chromatography (HPLC) with UV detection. Average percentage recovery of cytochrome *c* spiked with control rat brain mitochondria was $83.2 \pm 8.9\%$. Limits of detection and quantitation were 1 and 5ng, respectively. The results showed that a single dermal dose of a combination of DEET+ permethrin significantly increased the release of brain mitochondrial cytochrome *c* starting 24h after treatment. DEET and permethrin alone did not affect the release of cytochrome *c*. The results indicate that combined exposure to DEET and permethrin might induce the apoptotic processes in rat brain as seen by the release of cytochrome *c*

Introduction

Generation of reactive oxygen species could lead to an early release of mitochondrial cytochrome *c*, that is associated with inhibition of mitochondrial respiration and stimulation mitochondrial superoxide production (Cai and Jones, 1998). Further, the release of cytochrome *c* from mitochondria as a result of cell death leads to caspase activation (Abu-Qare and Abou-Donia, 2000a; Kuwana et al, 1998). Cells undergoing

apoptosis exhibit an elevation of cytochrome c in the cytosol and a corresponding decrease in the mitochondria (Yang et al, 1997, Finucane et al, 1999; Steemans et al, 1998). Bossy-Wetzel et al (1998) reported that cytochrome *c* release from mitochondria is an early event in the apoptotic process induced by UVB irradiation or saturosporine treatment in CEM or HeLa cells. In a cell-free apoptotic system, mitochondria were shown to spontaneously release cytochrome *c* (Kluck et al, 1997). Further, Schuler et al (2000) reported that *in vitro* p53 tumor suppressor gene activates the apoptotic machinery through induction of the release of cytochrome *c* from mitochondrial inter-membrane space; and that cell death was accompanied by caspase activation and cleavage of caspase substrates. Gorman et al., (1999) demonstrated a close correlation between the time course of cytochrome *c* release from the mitochondria and caspase-3-activation during colchicine-induced apoptosis of cerebellar granule cells, while Finucane et al (1999) indicated that the mitochondrial release of cytochrome *c* represents a critical step in cell death.

Levels of cytochrome *c* in cells and cellular tissues have been determined by the immunoblotting techniques, using a monoclonal antibody to cytochrome *c* (Zhuang and Cohen, 1998; Bossy-Wetzel and Green, 1999; Bobba et al, 1999; Li et al, 1999; Denecker et al, 2000; Fujii et al, 2000). HPLC techniques with UV detection at 393 nm have been recently used for determination of mitochondrial cytochrome *c* (Picklo et al, 1999). This method has advantages over classical immunoblotting method which has shortcomings such as, (1) quantification of an antigen by immunoblot is limited technically, (2) It may be complicated by incomplete transfer of the antigen to the blotting membrane, (3) masking of the antigen by cellular protein, (4) over-development or underdevelopment of

the blot, non-linearity of the signal, (5) impracticality of large number of samples (Picklo et al, 1999).

Permethrin is a pyrethroid insecticide effective against mites, head lice (Fraser, 1994). DEET is applied as an insect repellent on the skin against mosquitoes, sand flies, ticks and fleas (Brown and Hebert, 1997). Both compounds are widely used inside homes and in public places (Burgess, 1992).

Permethrin modifies sodium channels to open longer during a depolarization pulse (Narahashi, 1985). Previous studies showed that DEET has direct adverse effects on the nervous system in laboratory animals resulting in spongiform myelinopathy in the brain stem with signs including ataxia, seizures, coma and death (Verschoyle et al, 1990). Further, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Edwards and Johnson, 1987; Roland et al., 1985). Combined exposure to permethrin and DEET enhanced neurotoxicity in hens (Abou-Donia et al, 1996), and increased mortality in rats (McCain et al, 1997). Published reports implicated exposure to DEET and permethrin in the Gulf War Veterans Illnesses (Abou-Donia et al, 1996; Haley et al, 1999; Haley and Kurt, 1997; Hodgson and Kipen, 1999; Hoy et al, 2000).

No published reports have examined possible induction by DEET and permethrin, alone or in combination on the release of cytochrome *c* into cytosol. In this study, the release of rat brain mitochondrial cytochrome *c* was determined after a single dermal dose of DEET, and permethrin, alone and in combination using reversed phase-high performance liquid chromatography method with UV detection at 393 nm.

Materials and Methods

Chemicals

Rat heart cytochrome *c* (97%), and phenylmethylsulfonyl fluoride (>99%) were obtained from Sigma Co Inc., (St.Louis, MO). DEET (\geq 97%, *N,N*-diethyl-*m*-toluamide) was purchased from Aldrich Chemical Co, Inc. (Milwaukee, WIS), while permethrin (99%, 3-(2,2-dichloroethyl)-2,2-dimethylcyclopropane carboxylic acid (3-(3-phenoxyphenyl)methyl ester) was obtained from Chem Service, Inc. (West Chester, PA). Water and acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky).

HPLC System

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E Multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector and a Waters 2487 Dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2cm \times 4mm, 5 μ m, Supelco Park, Bellefonte, PA), and a reversed-phase C₁₈ column μ BondapakTM C₁₈ 125A° 10 μ m, 3.9 \times 300 mm were used, (Waters Corporation, Milford, MA)

Calibration curve, recovery and limits of detection

A standard calibration curves for concentrations between 0.5-50 μ g/ml of cytochrome *c* were obtained under the described HPLC conditions. The detection limits were determined as the lowest concentration that can be detected taking into consideration a 1:3 baseline: peak signal ratio. Recoveries of the cytochrome *c* from control rat brain mitochondria samples were determined for concentrations of 0.5-50 μ g/ml.

Experimental animals

Sprague Dawley rats (250-300 g) were purchased from Zivic Miller (Zelienople, PA). The untreated animals were kept in a 12-h light/dark cycle (temperature 21-23°C) and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purine Co., St. Louis, MO) as well as tap water. Animal care was conducted according to institutional guidelines.

Animals treatment

Permethrin and DEET were dissolved in 70% ethanol. A single dose of 1.3 mg/kg of permethrin and a single dose of a 400 mg/kg of DEET were applied with a micropipette (1ml/kg) to an unprotected 1 cm² area of pre-clipped skin on the back of each rat. A group of five animals was used for each time point. A combined single dermal dose of 1.3 mg/kg of permethrin, followed by a single dermal dose of 400 mg of DDET was also applied. The dosage selected represents real life exposure of each chemical as determined by US Department of Defense (Personal Communications). Control animals were treated with equal volume of 70% ethanol (1 ml/kg) and kept under similar conditions as treated rats. A group of five animals were anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ) and sacrificed by heart exsanguinations at time intervals of 0.5, 1, 2, 4, 8, 16, 24, 48, or 72 h after dosing. Rat brain was removed, plotted dry and washed in saline, then stored at -70°C prior to analysis by HPLC.

Isolation of brain mitochondrial cytochrome c

Mitochondria samples from rat brain were isolated using a modified method of Clark and Nicklas (1970). Rat brain (500 mg) was homogenized in 4 volumes of buffer (0.32 M sucrose, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin) in Potter-

Elvehjem homogenizer at 4°C. The homogenate was centrifuged at 1,000g for 20 min and the supernatant was centrifuged at 12,000g for 25 min at 4°C. The resulting supernatant was centrifuged again at 100,000g for 30 min to assure removal of all mitochondria. The resulting supernatant (cytosolic fraction) was then injected into HPLC for analysis of the release of cytochrome *c* into the cytosol. Protein content was estimated by the procedure of Smith et al. (1985).

Analysis

A volume of 10 μ l of the extracts was injected into the HPLC system as described above. The mobile phase consisting of 0.1 % (v/v) trifluoroacetic acid in water (pH 4.5) (solvent A) and acetonitrile (solvent B) was delivered at a flow rate of 1 ml/min with a linear increase of solvent B from 20% to 60% within 8 min. The column was washed after each run with 60% acetonitrile in 0.1% fluoroacetic acid for 5 min, then equilibrated with the mobile phase for 3 min. The eluents were monitored by detection at 393 nm. The chromatographic analysis was performed at ambient temperature.

Statistical Analysis

Analysis of Variance using GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA) was used to determine if the difference between treated and control is statistically significant. $P < 0.05$ was considered statistically different.

Results

Linearity, recovery and detection limits

A calibration standard curve for cytochrome *c* was obtained for a concentration range between 0.5-50 μ g/ml (5-500ng). Percentage average recovery of cytochrome *c* from rat

brain mitochondria was $83.2\pm8.9\%$. Limits of detection and quantitation of rat cytochrome *c* were 1ng and 5ng, respectively.

HPLC analysis

Figures. 1a and 1b represent the chromatograms of standard and rat brain mitochondrial cytochrome *c* under described HPLC conditions. Retention time of cytochrome *c* was 9.2 min.

Levels of cytochrome *c* in control and treated rats

Combined exposure to a single dermal dose of DEET and permethrin caused significant increase in the release of mitochondria cytochrome *c* 24h after dosing (Fig 2). A single dermal dose of DEET or permethrin did not produce statistically significant effect on the release of cytochrome *c* from brain mitochondria (Fig 2) over the time course of treatment. The mean (\pm SD) level of the released rat brain mitochondrial cytochrome *c* was 0.69 ± 0.1 , 1.1 ± 0.1 , 0.71 ± 0.06 , and 1.7 ± 0.08 μ g/mg mitochondria protein after 24hr after dosing of ethanol, DEET, permethrin, and a combination of DEET and permethrin, respectively.

Statistical analysis

A combined dose of DEET and permethrin caused significant induction of cytochrome *c* compared to control starting 24hr until 72hr of dosing (Fig.4). On the other hand there was no significant difference between DEET or permethrin treatment and ethanol treated rats.

Discussion

Our results indicate that combined dermal administration of DEET and permethrin to rats significantly induced the release of rat brain mitochondrial cytochrome *c* starting 24h

after dosing. In previous studies, the release of cytochrome *c* has been correlated with induction of the apoptotic processes. Martino et al (2000), reported that during the process of apoptosis, cytochrome *c* is released from mitochondria into the cytosol where it helps to activate the caspases, a family of killer proteases. Heiskanen et al (1999) detected the release of cytochrome *c* from mitochondria into cytosol in cells undergoing apoptosis. Further, the release of cytochrome *c* was rapid and complete during hypoxia/reoxygenation of cultured kidney cells, resulting in caspase activation whereas cells that lack cytochrome *c* underwent apoptosis after reoxygenation (Saikumar et al. (1998). Kannan et al (2000) reported that the organochlorine insecticide endosulfan induced the release of cytochrome *c* in human T-leukemic cell lines. Factors that inhibit or block the release of cytochrome *c* protect the cells from apoptosis. Kharbanda et al. (1997) reported that Bcl-xL inhibited the accumulation of cytosolic cytochrome *c*, and prevented cell apoptosis in response to genetic stress. Our results are in agreement with previous studies reported that chemicals induced release of cytochrome *c*-dependent mechanisms. *In vitro* incubation of purified mitochondria with pyrrolidine dithiocarbamate directly induced cytochrome *c* release from the intermembrane space (Della Ragione et al, 2000). Mirkes and Little (2000) showed that the teratogen 4-hydroperoxycyclophosphamide and heat shock induced the release of cytochrome *c* from mitochondria in 9 day old mouse embryos. Further, exposure to phosphatidyl serine caused the release of cytochrome *c* and decreased transmembrane potential in cells (Denecker et al., 2000). Li et al. (1999) also reported that apoptotic cell death induced by beta-lapachone, a potential anti-cancer drug, was preceded by a rapid release of cytochrome *c*, followed by activation of caspase 3 in apoptotic cell death. Furthermore,

treatment of HL-60 cells with staurosporine (STS) induced mitochondrial cytochrome *c* efflux into the cytosol, which was followed by caspase-3-activation and apoptosis (Han et al., 1998).

Environmental factors and stress play role in the release of cytochrome *c*. Balk et al (1999) showed that heat (55°C) induced programmed cell death in cucumber plants through translocation of cytochrome *c* from mitochondria to cytosol. Further, Chen et al (2000) reported that ionizing radiation induced rapid release of cytochrome *c* from mitochondria into the cytoplasm that activated caspases, and resulted in the breakdown of the amino acid sequence DEVD that was involved in apoptosis. Exposure of human cervical carcinoma C33A cells to the anti-cancer drug *N*-(4-hydroxyphenyl) retinamide induced cytochrome *c* release from mitochondria to cytoplasm, increased reactive oxygen species(ROS), activated caspase-3, and caused transition membrane permeability (Suzuki et al, 1999).

Significant induction of the release of cytochrome *c* has been detected 24h after combined exposure to DEET and permethrin. The lack of an induction in the release of cytochrome *c* at early times following administration suggests slow absorption of the test compounds that resulted in concentrations below the threshold levels of cytochrome *c* release. In this study, the dose of DEET and permethrin was as a real-life exposure level determined by U.S. Department of Defense (Personal Communications).

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Figure legends

Fig. 1a HPLC chromatogram of cytochrome *c* standard

Fig. 1b HPLC chromatogram of cytosolic fraction of rat brain treated with a combined dose of DEET and permethrin.

Fig. 2 Levels of rat brain cytosol cytochrome *c* ($\mu\text{g}/\text{mg}$ mitochondrial protein) following a single dermal dose of 400 mg/kg of DEET, 1.3 mg/kg of permethrin, alone and in combination. Asterisk indicates significantly different from control, $p<0.05$.

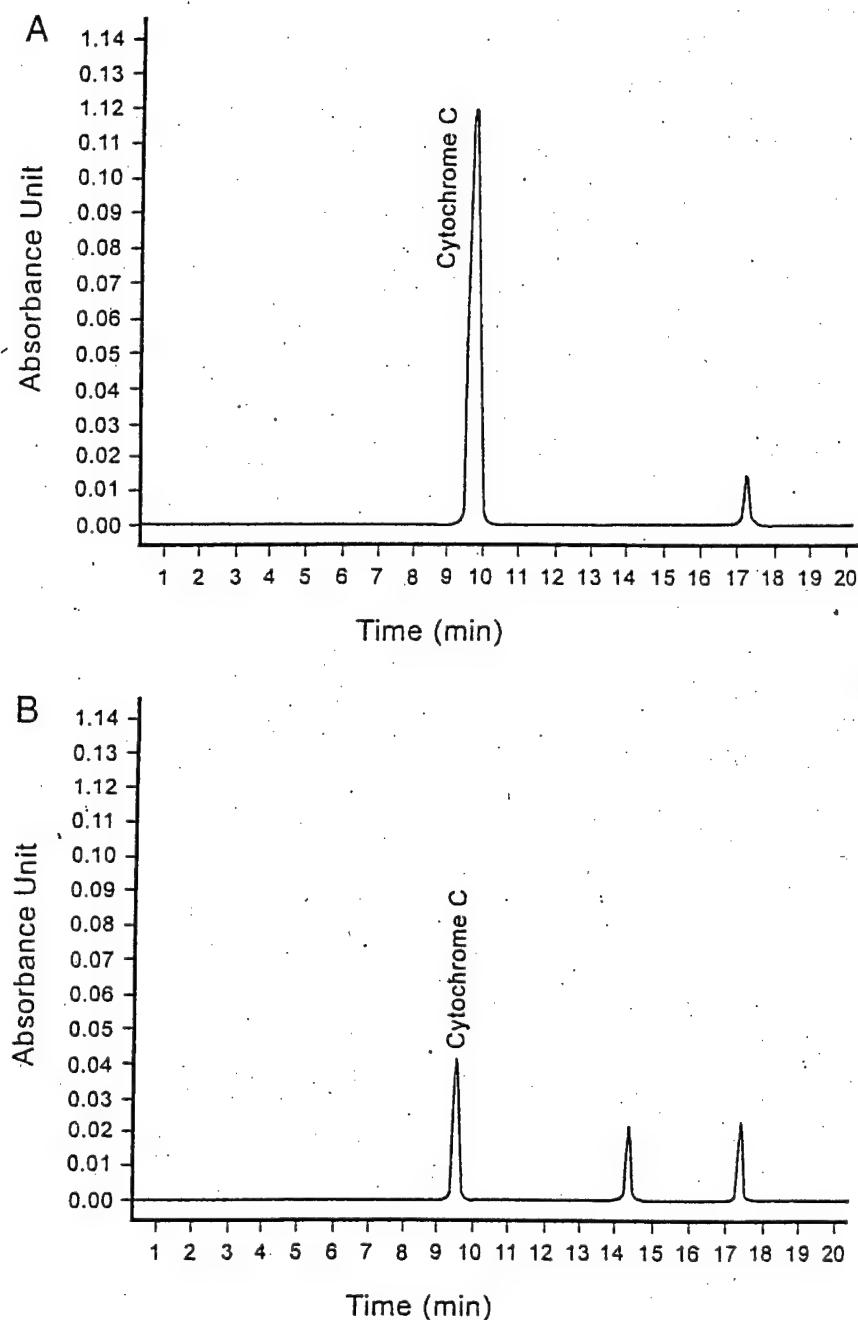


Figure 1

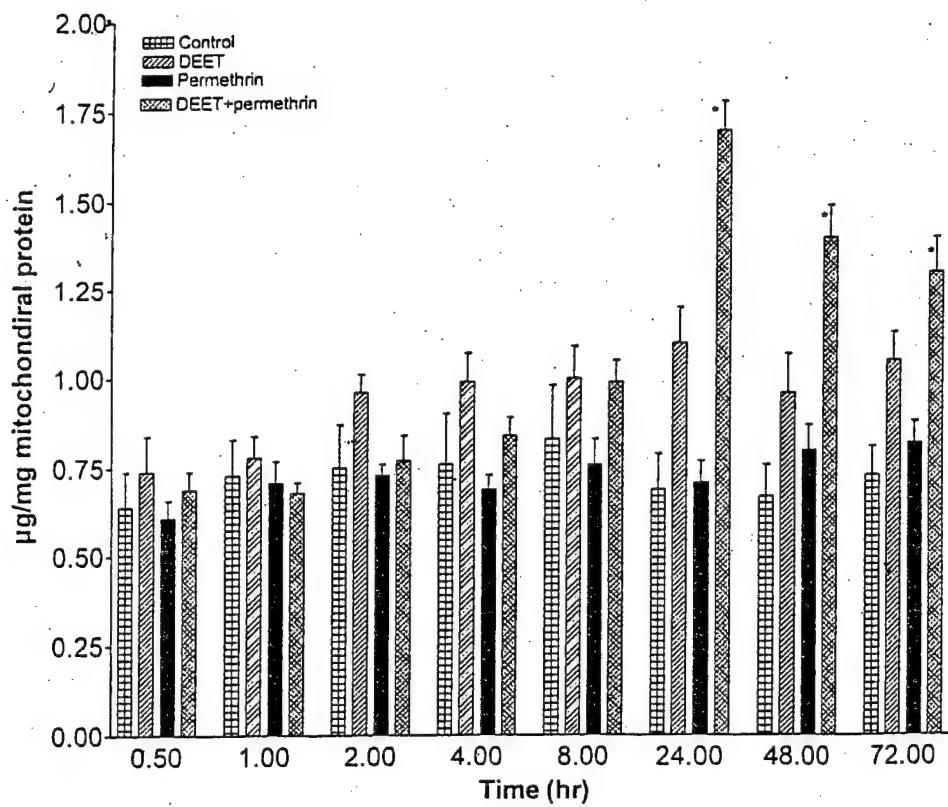


Figure 2

3. Effect of Pyridostigmine Bromide, DEET, and/or Permethrin On Urinary Excretion of 3-Nitrotyrosine, a Marker for Oxidative Stress

Abstract

In this study we determined levels of 3-nitrotyrosine in rat urine following administration of a single oral dose of 13 mg/kg of pyridostigmine bromide (PB, 3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide), a single dermal dose of 400 mg/kg of DEET and a single dermal dose of 1.3 mg/kg of permethrin, alone and in combination. Urine samples were collected from five treated and five control rats at 4, 8, 16, 24, 48, and 72 h following dosing. Solid phase extraction coupled with high-performance liquid chromatography (HPLC) with UV detection at 274nm was used for the determination of tyrosine, and 3-nitrotyrosine. A single oral dose of PB and a single dermal dose of DEET or their combination significantly ($P<0.05$) increased levels of 3-nitrotyrosine starting 24h after dosing compared to control urine samples. Maximum increase of 3-nitrotyrosine was detected 48 h after combined administration of PB and DEET. Ratio of 3-nitrotyrosine to tyrosine in urine excreted 48h after dosing was 0.19 ± 0.04 , 0.20 ± 0.05 , 0.28 ± 0.03 , 0.32 ± 0.04 , 0.19 ± 0.05 , 0.42 ± 0.04 , 0.27 ± 0.03 , 0.36 ± 0.04 , 0.48 ± 0.04 following administration of water, ethanol, PB, DEET, permethrin, PB+DEET, PB+permethrin, DEET+permethrin, and PB+DEET+permethrin, respectively. The results indicate that oral dose of PB, and dermal administration of DEET, alone and in combination could generate free radical species, thus increase levels of 3-nitrotyrosine in rat urine. Induction of 3-nitrotyrosine, a marker of oxidative following exposure to these compounds could be significant in understanding the proposed enhanced toxicity following combined exposure to these compounds.

Introduction

Cells generate nitrogen oxide (NO) and other reactive oxygen species such as superoxide anion. Their reaction forms peroxynitrite (Kaur and Halliwell, 1994; Hensley et al., 2000). Peroxynitrite reacts with tissue protein tyrosine to form 3-nitrotyrosine that is used as a marker for oxidative stress (Roberts et al., 1998). Several reports have been published indicating induction of 3-nitrotyrosine following exposure to chemicals (Pennathur et al., 1999; Salman-Tabchen et al. 1995).

Several methods have been used for the determination of 3-nitrotyrosine in biological matrices (Herce-Pagliai et al., 1998; Rimbach et al., 1999). Among these methods are using high performance liquid chromatography (HPLC) with UV, photodiode array or electrochemical detection (Salman-Tabchen et al., 1995; Kamisaki et al., 1996; Fukuyama et al., 1997; Kaur et al., 1998; Tabrizi-Fard et al., 1999; Hirabayashi et al., 2000; Sodium et al., 2000; Van Dalen et al., 2000).

Pyridostigmine bromide (PB) is a drug used in the treatment of patients of myasthenia gravis-an autoimmune disease affecting the neuromuscular junction and producing weakness of voluntary muscles (Aquilinius and Hartvig, 1986), and is applied as a prophylaxis against nerve agents (Young and Evans, 1998; Abou-Donia et al., 1996). It inhibits acetylcholinesterase and butyrylcholinesterase in the peripheral nervous system (Abou-Donia et al, 1996; Abou-Donia et al, 2000). Permethrin is an insecticide effective against mites and head lice (Burgess et al, 1992). Permethrin modifies sodium channel to open longer during a depolarization pulse (Narahashi, 1985). DEET is applied as an insect repellent (Brown and Hebert, 1997). Previous studies showed that DEET has a direct effect on the nervous system in laboratory animals resulting in spongiform

myelinopathy in the brain stem with signs including ataxia, seizures, coma and death (Verschoyle et al, 1990). Furthermore, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Edwards and Johnson, 1987; Roland et al., 1985). The three compounds have been used during the Gulf War to protect veterans against nerve agents and biting insects (Young and Evans, 1998). Combined exposure to PB, permethrin and DEET enhanced neurotoxicity in hens (Abou-Donia et al, 1996) and increased mortality in rats (McCain et al, 1998). Published reports implicated combined exposure to these compounds that resulted in enhanced neurotoxicity in chickens, increased mortality in rats, or induced behavioral changes in the Gulf War Illnesses (Abou-Donia et al, 1996; Haley and Kurt, 1997; Wilson et al, 1998; Hoy et al, 2000). In this study, we present results of analysis of urine levels of 3-nitrotyrosine following administration of PB, DEET and permethrin, alone and in combination in rats.

Materials and methods

Chemicals and materials

Tyrosine, 3-nitrotyrosine and pyridostigmine bromide (98% PB, 3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide) (Figure. 1) were obtained from Sigma Co Inc., (St.Louis, MO). DEET ($\geq 97\%$, *N,N*-diethyl-*m*-toluamide) was purchased from Aldrich Chemical Co, Inc.(Milwaukee, WIS), while permethrin (99%, 3-(2,2-dichloroethyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was purchased from Chem Service, Inc. (West Chester, PA). Water and acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker, Inc. (Paris,

Kentucky). C₁₈ Sep-Pak Cartridges were obtained from Waters Corporation (Milford, MA).

Animals treatment and use

Sprague Dawley rats (200-240 g) were purchased from Zivic Miller (Zelienople, PA). The animals were kept in a 12-h light/dark cycle (temperature 21-23°C) for one week, and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purine Co., St. Louis, MO) and tap water. Animal care was conducted according to NIH guidelines. The RAC approval number is A612-99-12-2. A single dose of 13mg/kg of PB was applied orally. A single dose of 1.3 mg/kg of permethrin and of a 400 mg/kg of DEET were applied in ethanol with a micropipette to an unprotected 1 cm² area of pre-clipped skin on the back of each rat. A group of five animals was used for each time point. (The doses represent real-life exposure (Abu-Qare and Abou-Donia, 2000a; Abu-Qare and Abou-Donia, 2000b). Combined single dermal dose of 13 mg/kg of pyridostigmine bromide, and 1.3 mg/kg of permethrin, followed by a single dermal dose of 400 mg/kg of DEET was also applied. Five control rats were treated with equal volume of either water or ethanol and kept under similar conditions as treated rats. Urine samples were collected at 4, 8, 16, 24, 48, and 72 h after dosing. The samples were frozen at -20°C until analysis. A 2 ml sample of the collected urine at each time point was taken for analysis.

HPLC System

The liquid chromatographic system (Waters 2690 Separation Module) consisted of a Waters 600E Multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector and a Waters 2487 Dual λ absorbance detector (Waters Corporation, Milford, MA). A

guard column (Supelco, 2cm×4.0mm, 5 μ m, Supelco Park, Bellefonte, PA), and a reversed-phase C₁₈ column μ BondapakTM C₁₈ 125A° 10 μ m, 3.9×300 mm were used, (Waters Corporation, Milford, MA)

Calibration curve, recovery and limits of detection

The standard calibration curves for tyrosine and 3-nitrotyrosine were linear for concentrations of 0.1, 0.2, 0.4, 0.5, 1, and 10 μ g/ml. The calibration curves were generated using GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA). Detection limits were determined as the lowest concentration that can be detected taking into consideration a 1: 3 base line: peak signal ratio. Recoveries of the compounds from blank urine samples collected at 0-hr of the experiment were determined for concentrations of 0.1-10 μ g/ml. Known concentrations were spiked with control urine samples (0-hr samples) and analyzed as described under sample preparation. Amounts of tyrosine, and 3-nitrotyrosine were corrected based on the recovery obtained.

Sample preparation

A volume of 2.0 ml of the urine samples was acidified (pH 5.0) using 0.1M acetic acid, then loaded on a disposable C₁₈ Sep-Pak Vac 3cc (500mg) cartridges (Waters Corporation, Milford, MA) previously conditioned with 2 ml of methanol, and equilibrated using 2 ml of water and 2 ml of 0.03M phosphate dibasic buffer (pH 5.0) prior to use. After washing with 2 ml of water, the sample was eluted using 2×1 ml of methanol, the methanolic eluates were reduced to 500 μ l in small test tube using a gentle stream of nitrogen at room temperature, prior to analysis by HPLC. Urine samples were diluted to fit within the calibration curves.

Analysis of urine samples

A volume of 10.0 μ l of the extracts was injected into the HPLC system as described above. The mobile phase consisted of 84% water (adjusted to pH 3.0 using 0.1M acetic acid) and 16% methanol at flow rate of 0.60 ml/min. The eluents were monitored by UV detection at dual wavelengths of 274nm and 254nm. The chromatographic analysis was performed at ambient temperature. Amount of tyrosine and 3-nitrotyrosine was calculated and corrected based on the urine volume and total body weight of the animal.

Statistical Analysis

Analysis of Variance (ANOVA) using a GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA) was used to determine if the difference between treated and control is significant. A value of $P<0.05$ was considered statistically significant. Values are expressed as mean \pm SD of five replicates.

Results

Linearity, Recovery and limits of detection

Calibration standard curves for tyrosine and 3-nitrotyrosine were linear for concentrations ranging between 0.1-10 μ g/ml, with correlation coefficients of $r^2 >0.998$. Average percentage recoveries of tyrosine and 3-nitrotyrosine ranged from 78.8%-92.5%. Limit of detection of tyrosine, and 3-nitrotyrosine was 0.2ng (20ng/ml).

HPLC analysis

Figures.2a&2b show chromatograms of standard and urine samples of tyrosine, and 3-nitrotyrosine obtained under described HPLC conditions. Retention times were 4.4 min, and 7.1 min for tyrosine, and 3-nitrotyrosine, respectively.

Levels of L-tyrosine and 3-nitrotyrosine

A single oral dose of 13 mg/kg of PB, and a single dermal dose of 400 mg/kg of DEET caused significant increase in the urinary excretion of 3-nitrotyrosine when applied, alone or in combination after 24h of dosing (Fig.3). Maximum induction was detected 48 h after dosing. Amounts of tyrosine and 3-nitrotyrosine were corrected according to total volume of the urine and body weight of the animal and the recovery from urine samples.

Statistical analysis

There was a statistically significant increase in the amount of 3-nitrotyrosine after 24 h of administration of PB, DEET, and their combination ($P<0.05$). Administration of a single permethrin did not cause significant effect on level of 3-nitrotyrosine compared to control urine ($P>0.05$).

Discussion

The results indicate that a single oral dose of (PB) and a single dermal administration of DEET, alone and in combination significantly increased rat urinary excretion of 3-nitrotyrosine starting 24h after dosing. In this study we used a real-life exposure levels as determined by U.S Department of Defense (Personal Communications). Induction of 3-nitrotyrosine has been widely studied as marker of oxidative stress. Several published reports implicated its induction with diseases. A variety of chemicals have been shown to increase its levels in different biological matrices. Pennathur et al. (1999) reported that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) increased levels of 3-nitrotyrosine in brain tissues. Further, Imam et al. (1999) reported that administration of methamphetamine in mice resulted in the significant formation of 3-nitrotyrosine in the striatum. It is believed that formation of 3-nitrotyrosine proceeds through free radical

mechanism (Niwa et al, 1999; Pannala et al, 1998). It might that PB and DEET induced generation of free radicals, thus formation of peroxynitrite. Then increased of 3-nitrotyrosine. Involvement of free radicals in the formation 3-nitrotyrosine was detected following irradiation of *S*-nitrosoglutathione in the presence of tyrosine. Pannala et al. (1998) reported that hydroxycinnamates antioxidants decreased peroxynitrite-mediated nitration of tyrosine *in vitro*. Further, Niwa et al. (1999) showed that the antioxidants caffeic acid and sinapinic acid, inhibited formation of 3-nitrotyrosine in protein treated with peroxynitrite. An increased concentration of 3-nitrotyrosine has been implicated in various diseases that resulted in oxidative stress conditions (Halliwell et al., 1999; Schwemmer et al., 2000; Gole et al., 2000). An increased plasma level of 3-nitrotyrosine was considered as a biomarker of oxidative stress in chronic renal failure patients with septic shock (Fukuyama et al., 1997). Further, Kaur and Halliwell (1994) reported that blood serum from patients with the inflammatory joint disease rheumatoid arthritis contained 3-nitrotyrosine, while there was no detection of 3-nitrotyrosine in blood serum of normal subjects. Tohgi et al. (1999) reported an increase by more than six fold in the level of 3-nitrotyrosine and of 3-nitrotyrosine/tyrosine ratio in neurons of patients with Alzheimer's disease compared to normal controls of similar age.

The results show that exposure of rats to a single dermal dose of 1.3 mg/kg of permethrin did not cause significant increase of urinary excretion of 3-nitrotyrosine, suggesting that oxidative stress was similar to that of control levels, while an oral dose of PB and dermal dose of DEET, alone or in combination significantly increased levels of 3-nitrotyrosine in rat urine.

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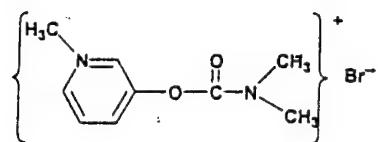
Figure legends

Fig .1 Structures of pyridostigmine bromide, DEET (*N,N*-diethyl-*m*-toluamide) and permethrin, tyrosine, and 3-nitrotyrosine.

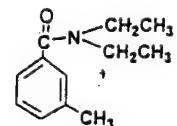
Fig. 2 (a) HPLC chromatogram of standard solutions of A) tyrosine, C) B) 3-nitrotyrosine.

(b) HPLC chromatogram of urine samples following a single oral dose of pyridostigmine bromide and a single dermal dose of DEET (*N,N*-diethyl-*m*-toluamide) and permethrin, A) tyrosine and B) 3-nitrotyrosine

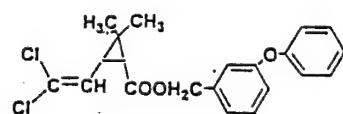
Fig. 3 Ratio of 3-nitrotyrosine to tyrosine in urine samples collected following a single oral dose of pyridostigmine bromide and a single dermal dose of DEET (*N,N*-diethyl-*m*-toluamide) and permethrin, alone and in combination. Ratio of 3-nitrotyrosine to tyrosine in control urine samples collected at (0-hr) was 0.15 ± 0.04 . * Significantly different from controls at $p<0.05$. # Significantly different from treated with individual compound at $p<0.05$.



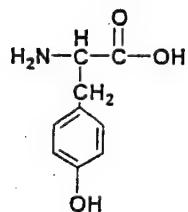
Pyridostigmine bromide



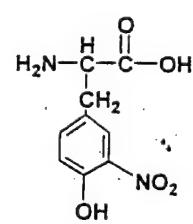
DEET (*N,N*-diethyl-*m*-toluamide)



Permethrin



Tyrosine



3-Nitrotyrosine

Figure 1

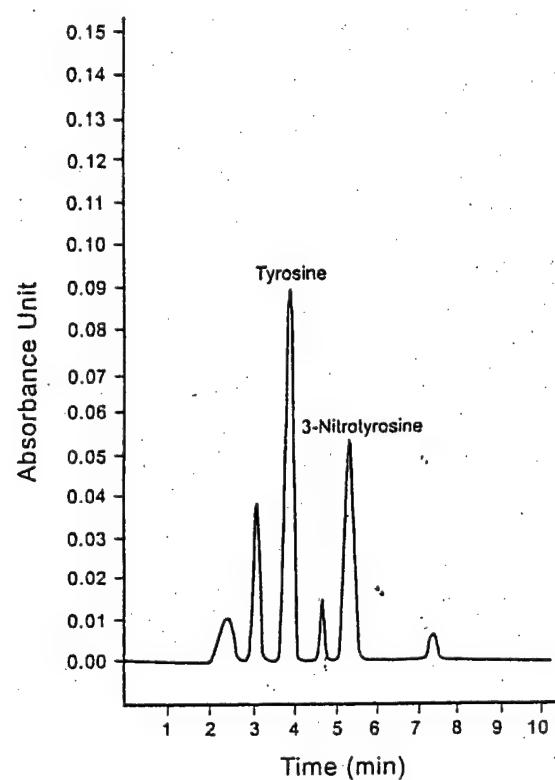
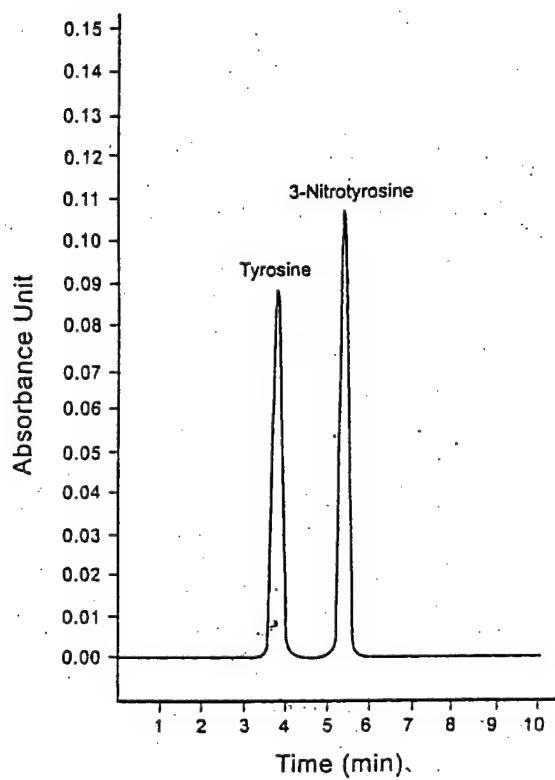


Figure 2

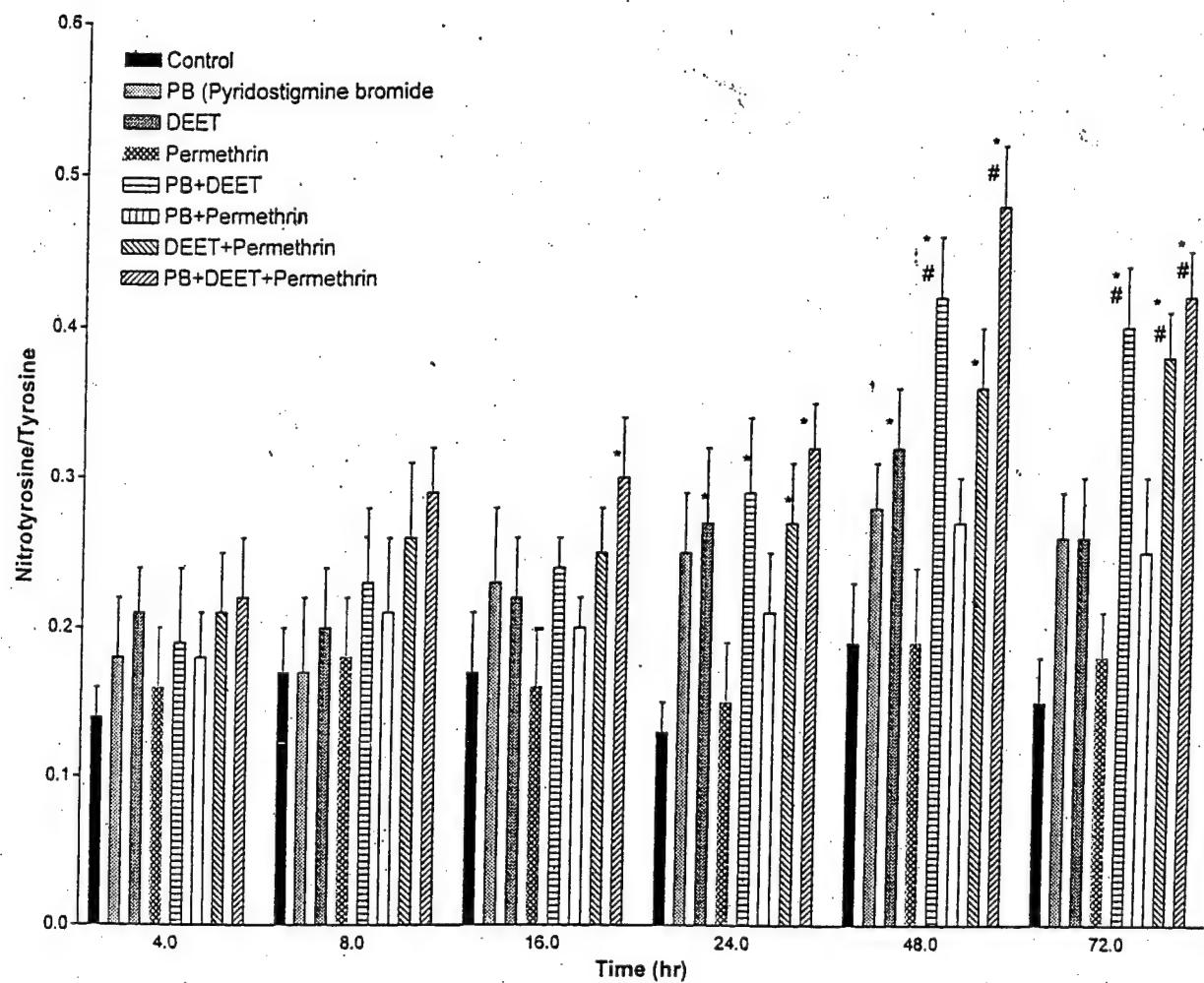


Figure 3

4. Effect of DEET and Permethrin, Alone or In Combination on the Urinary Excretion of 6 β -hydroxycortisol, A Marker for Hepatic CYP3A Induction in Rats

Abstract

In this study, the ratio of 6 β -hydroxycortisol (6 β -OHF) to free cortisol (F) was determined in urine following a single dermal dose of 400 mg/kg of DEET (*N,N*-diethyl-*m*-toluamide), and 1.3 mg/kg of permethrin, alone and in combination in rats. Urine samples were collected at 2, 4, 8, 16, 24, 48, and 72 hour after application. Recoveries of 6 β -OHF and cortisol (F) from control urine samples were between 75-85% with limits of detection at 30 and 10 ng/ml for cortisol and 6 β -OHF, respectively. A single dermal dose of DEET alone, and in combination with permethrin significantly increased urinary excretion of 6 β -hydroxycortisol 24 hours after dosing. Permethrin did not significantly alter the urinary excretion of 6 β -hydroxycortisol. These results indicate that DEET, alone and in combination with permethrin, increased urinary excretion of 6 β -OHF in rats following a single dermal dose application in rats.

Introduction

The use of biomarkers in the assessment of toxicological impact of chemicals is becoming increasingly significant (Timbrell, 1999; Abu-Qare and Abou-Donia, 2001). Cortisol is a natural hormone that helps cope with stress. Its levels in the plasma or urine are correlated with various diseases including depression (Carroll et al., 1976). Cortisol hydroxylation to 6 β -hydroxycortisol is catalyzed by hepatic CYP3A enzyme. The ratio of urinary (6 β -OHF) to free cortisol (F) has been reported to be a specific marker for CYP3A4 induction in *in vivo* studies of humans (Lucas et al., 1998). In rat

liver fractions *in vitro*, cortisol was shown to be metabolized to 6 β -OHF (Abel et al., 1993). Several studies reported induction of hepatic CYP3A following exposure to exogenous chemicals (Reichel et al., 1997; Koup et al., 1998; Kovacs et al., 1998; Tang et al., 1999). Examples of this induction include treatment of healthy male volunteers with (1) the drugs olanzapine and carbamazepine, alone or in combination (Lucas et al., 1998), (2) treatment of healthy males with the drug rifapentine (Reichel et al., 1997), (3) treatment of subjects with multiple doses of troglitazone (Koup et al., 1998), and (4) after 14 days of treatment of subjects with the drug ticlopidine (Boissonnat et al., 1997).

Furthermore, the ratio of (6 β -OHF)/cortisol (F) has been used as a parameter to detect physiological, biochemical or genetic population differences. A difference in the ratio of urine excretion has been detected between (a) Asian and Caucasian women (Lin et al., 1999), (b) between mature and pre-mature neonates, and (c) women during treatment with contraceptive drugs (Kuhnz and Lofberg, 1995).

Permethrin is a pyrethroid insecticide effective against head lice (Burgess et al., 1992). DEET is applied as an insect repellent (Brown and Hebert, 1997; Stinecipher and Shah, 1997). Both compounds have been used by U.S military personnel during the Persian Gulf War to protect veterans against mosquitoes and biting insects (Young and Evans, 1998). Absorption of DEET and permethrin was rapid following dermal administration in rat and human. Both compounds were metabolized through similar pathways in humans and rats (Abu-Qare and Abou-Donia, 2000a; Seliem et al., 1995; Anadon et al., 1991). Permethrin modifies sodium channels to open longer during a depolarization pulse (Narahashi, 1985). Previous studies showed that DEET has a direct effects on the nervous system in laboratory animals resulting in spongiform myelinopathy in the brain

stem with signs including ataxia, seizures and coma followed by death (Verschoyle et al., 1990). Furthermore, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Edwards and Johnson, 1987; Roland et al., 1985). In recent studies Abu-Qare and Abou-Donia (2000b) and Abu-Qare et al. (2001) reported that dermal dose of DEET increased urinary levels of 8-hydroxy-2-deoxyguanosine and 3-nitrotyrosine, markers of oxidative stress in rats. No available information reported on CYP induction following exposure to DEET. Combined exposure to permethrin and DEET enhanced neurotoxicity of each individual chemical in hens (Abou-Donia et al, 1996), and increased mortality in rats (McCain et al, 1998). Several reports implicated exposure to DEET and permethrin resembled Gulf War illness symptoms (Abou-Donia et al, 1996; Haley and Kurt, 1997; Kurt, 1998; Shen, 1998; Wilson et al, 1998; Hoy et al, 2000).

Studies to date indicate that the CYP3A4 P450 is a dominant oxidative enzyme in human drug metabolism involved in the metabolism of more than 50% of all drugs (Wrighton and Stevens, 1992; Li et al, 1995). CYP3A4 is also involved in metabolism of a large number of xenobiotics including pesticides. The main purpose of the study is to examine effect of combined exposure to these compounds on increased urinary levels of (6 β -OHF). It is the first study to report an increase of (6 β -OHF) following exposure to DEET alone and in combination with permethrin. Such increase could be significant following concurrent administration of common drugs or exposure to other xenobiotics that are metabolized through the same isoform of P450.

Materials and methods

Chemicals and materials

6 β -hydroxycortisol (98%), and cortisol (98%) were obtained from Sigma Co Inc., (St Louis, MO). DEET (\geq 97%, *N,N*-diethyl-*m*-toluamide) was purchased from Aldrich Chemical Co, Inc.(Milwaukee, WI), while permethrin (99%, 3-(2,2-dichloroethyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was obtained from Chem Service, Inc. (West Chester, PA). Water (HPLC grade), ethyl acetate and acetonitrile were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky). C₁₈ Sep-Pak^R Cartridges were obtained from Waters Corporation (Milford, MA).

HPLC System

The liquid chromatographic system (Waters 2690 Separation Module), consisted of Waters 600E Multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector and a Waters 2487 Dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2cm \times 4 mm, 5 μ m, Supelco Park, Bellefonte, PA), and a reversed-phase C₁₈ column μ BondapakTM C₁₈ 125A° 10 μ m, 3.9 \times 300 mm were used, (Waters Corporation, Milford, MA)

Calibration curve, recovery and limits of detection

Standard calibration curve of a concentration between 50-500 ng/ml of 6 β -hydroxycortisol and cortisol was obtained under the HPLC conditions described. The detection limits were determined as the lowest concentration that can be detected, taking into consideration a 1: 3 base line: peak signal ratio.

Recoveries of the chemicals from urine samples were determined for concentrations of 50 ng/ml-500 ng/ml.

Experimental animals

Male Sprague Dawley rats (250-300 g) were purchased from Zivic Miller (Zelienople, PA) . The untreated animals were kept in a 12-h light/dark cycle (temperature 21-23°C) and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purina Co., St. Louis, MO) and tap water. Animal care was conducted according to institutional guidelines and reviewed by animal care committee.

Animal treatment

Permethrin and DEET were dissolved in 70% ethanol. A single dose of 1.3 mg/kg of permethrin and a single dose of a 400 mg/kg of DEET (real-life exposure dose as determined by US Department of Defense (personal communication) was each applied with a micropipette (1ml/kg) to an unprotected 1 cm² area of pre-clipped skin on the back of each rat. The chemicals were applied separately each in 0.1 ml 70% ethanol. DEET applied first (on half of the area) then permethrin on the second half. A group of five animals was used for each time point. Combined treatment was carried out by applying a single dermal dose of 1.3 mg/kg of permethrin followed by a single dermal dose of 400 mg of DEET. Five control rats were treated with equal volume of 70% ethanol (1ml/kg) and kept under similar conditions as treated rats.

Animals handling

After dosing, each rat was placed in a metabolic cage, and urine samples were collected from each group of five animals at 2, 4, 8, 16, 24, 48, and 72 h after dosing. The samples were frozen at -20°C until analysis.

Sample preparation

A volume of 2.0 ml of the urine samples was acidified (pH 5) using 0.1N acetic acid, then applied on a disposable C₁₈ Sep-Pak Vac 3cc (500mg) cartridges (Waters Corporation, Milford, MA) previously conditioned with 2 ml of acetonitrile, and equilibrated using 2 ml of water prior to use. After washing with 4 ml of water, the sample was eluted using 2×2 ml of ethyl acetate, the eluates were reduced under vacuum at <40°C to 500 µl, prior to analysis by HPLC.

Analysis

A volume of 20 µl of the extracts was injected into the HPLC system as described above. The mobile phase consisting of 0.01% acetic acid (pH adjusted to 4.5) (solvent A), and acetonitrile (solvent B), was delivered at a flow rate of 0.7 ml/min with a linear increase of solvent B from 15% to 75% over 10 min. The system was equilibrated for 3 min, giving a total chromatographic run time of 13 min. The eluents were monitored by UV detection at 243 nm. The chromatographic analysis was performed at ambient temperature.

Statistical Analysis

Analysis of Variance (two-tailed) using GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA) was used to determine if the difference between treated and control is significant. Two-tailed analysis of variance was performed. A value of (p<0.05) was considered significant.

Results

Linearity, Recovery and detection limits

Calibration standard curves for 6β -hydroxycortisol and cortisol were obtained for concentrations range between 50 -500 ng/ml (Fig.1). Recovery of 6β -hydroxycortisol and cortisol from urine samples was determined. Percentage average recoveries were $74.8\pm9.2\%$, and $85.5\pm6.8\%$ for 6β -hydroxycortisol and cortisol, respectively. Limits of detection of 6β -hydroxycortisol and cortisol were 30, and 10 ng/ml, respectively.

HPLC analysis

Figure 2 shows the chromatogram of urine samples of 6β -hydroxycortisol and cortisol under described HPLC conditions. Retention times were 7.2 min, and 9.3 min for 6β -hydroxycortisol and free cortisol, respectively

Levels of 6β -hydroxycortisol and cortisol in urine

A single dermal dose of 400 mg/kg of DEET alone and in combination with dermal dose of 1.3 mg/kg of permethrin caused significant increase in the urinary excretion of 6β -hydroxycortisol at 24, 48, and 72 h after dosing (Fig.3). There was no significant effect within 16 h of treatment. A single dermal dose of 1.3 mg/kg of permethrin did not produce statistically significant effect on the level of 6β -hydroxycortisol in rat urine over the time course of treatment. Urinary cortisol concentrations are shown in Figure.4.

Discussion

The results indicate that dermal administration of DEET in rats increased urinary excretion of 6β -hydroxycortisol. Both dermal doses of DEET when applied alone or in combination with permethrin caused similar induction after 24 h of administration. The lack of an increase in the excretion of 6β -hydroxycortisol in the early time following

administration could be due to the fact that the amount of DEET absorbed at that time was below the threshold level. The increase of 6 β -OHF was reported as ratio to cortisol and compared to control rats. In this study, the dose of DEET used was a real-life exposure level as determined by US Department of Defense (personal communication).

In this study, recovery of 6 β -hydroxycortisol, and free cortisol from control urine samples were 75% and 85%, respectively. This is consistent with previous reports; Lykkesfeldt et al. (1994) reported a recovery of 70.8% and 90.6% from urine for 6 β -hydroxycortisol, and cortisol, respectively. While Tang et al. (2000) reported recovery of cortisol from urine to be >65% when solid phase extraction followed by methanol. Furthermore, Hay and Mormede. (1997) reported recovery of 83% for cortisol from urine, while its recovery was between 92-95% from adult urine using HPLC-UV method (Lee, 1995).

Separation of 6 β -hydroxycortisol, and cortisol was achieved under the described HPLC condition without interference from urine constituents. Limits of detection reported from our method were 30 and 10 ng/ml for 6 β -hydroxycortisol and cortisol, respectively. In other methods the detection limits were reported to be 10ng/ml for cortisol using HPLC with UV detection (Hay and Mormede, 1997), and 1.5 ng/ml using GC-MS method (Furuta et al, 2000). Using HPLC with fluorescence detection decreased limits of detection to 0.68 and 0.95ng/ml, for 6-beta-OHF, and F, respectively (Inoue et al., 1994). Our ability to detect both chemicals in most samples analyzed (2 ml urine) proved that our method is adequate in measuring urinary ratios of 6 β -hydroxycortisol, and free cortisol as a biomarker.

The increase in the ratio of 6B-hydroxycortisol to cortisol in our experiment following DEET, and DEET+permethrin treatment compared to control is consistent with previous reports of rise in the ratios of 6 β -hydroxycortisol (6 β -OHF) to free cortisol (F) following an oral dose of the CYP3A inducer rifampicin in common marmosets (Tosuka et al, 1999). While Fleishaker et al. (1995) reported that 6 β -hydroxycortisol to cortisol ratios increased by a factor of 2.37 from baseline following treatment of healthy subjects volunteers with phenytoin. A 4 fold rise in the urinary excretion of 6 β -hydroxycortisol after treatment with rifampicin has been reported (Reichel et al, 1997). The results are in agreement with previous report of Kovacs et al. (1998) who found a significant increase in the 24 hr urinary excretion of 6 β -hydroxycortisol and the ratio of hydroxycortisol (6 β -OHF) to free cortisol (F) in subjects treated with the drug rifampicin. Furthermore, Tang et al (1999) reported a percentage increase in the morning spot and 24 h ratio of hydroxycortisol (6 β -OHF)/cortisol in the urine of healthy adult male volunteers following treatment with rifampicin. Boissonnat et al. (1997) reported that following 14 days of treatment with the drug ticlopidine, levels of 6 β -hydroxycortisol, increased in urine samples collected 24 h after the last dose.

Urinary excretion of 6 β -hydroxycortisol reached a peak level at 24 h after dermal dose of DEET alone, or in combination with permethrin and then remained unchanged. The levels of 6 β -hydroxycortisol excreted in urine following treatment with DEET was 188 ng/ml. In previous studies the reference range of urinary 6 beta-OHF was 187-1245 nmol/day in adults (20-26 years), and 103-915 nmol/day in the elderly (>61 years) (Lee, 1995). Reichel et al, (1997) reported that the levels of 6 β -hydroxycortisol were elevated from 2.1 (day 1) to 9.9 μ g/day/kg on day 13 following treatment of male volunteers with

rifampicin. The ratio of 6β -hydroxycortisol to cortisol 24 h after DEET treatment was 9.8 ± 3.6 . This is consistent with a report of a ratio between 15-10 of 6β -hydroxycortisol to cortisol following phenobarbital treatment in rats (Tang et al, 2000).

These results show that exposure of rats to a dermal dose of 1.3 mg/kg of permethrin not cause a significant increase in the ratio of urinary excretion of 6β -hydroxycortisol to free cortisol. The small dermal dose of 1.3 mg/kg of permethrin used and the slower dermal absorption of permethrin compared to DEET might have resulted in a small concentration below the threshold level for induction of urinary levels of 6β -hydroxycortisol. Rost et al. (1997) did not find significant differences in urinary levels of 6β -hydroxycortisol following treatments with therapeutic dose of lansoprazole. The levels of 6β -hydroxycortisol before and after treatment were 212, and 255 $\mu\text{g}/\text{day}$, respectively. Pichard-Garcia et al (2000) reported the use of 6β -hydroxycortisol (6β -OHF)/cortisol (F) ratios as a marker of CYP3A4 activity to assess the effect of the migraine drug eletriptan *in vitro* using human hepatocytes as inducer of CYPP3A4.

In summary, dermal application of DEET increased the ratios of 6β -hydroxycortisol to free cortisol in rats urine. Higher dose of permethrin might do so.

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Figure legends

Fig.1 Standard calibration curves of 6β -hydroxycortisol and cortisol. Each point is a means \pm SE of five replicates.

Fig.2 HPLC chromatogram of urine sample of rats treated with a single dermal dose of 400 mg/kg of DEET.

Fig.3 Excretion of 6β -hydroxycortisol in urine samples following a single dermal dose of 400 mg/kg of DEET, and 1.3 mg/kg of permethrin, alone and in combination in rats. *

Statistically different from control at ($P<0.05$). Each par is a means \pm SE of five samples.

Fig. 4 Excretion of cortisol in urine samples collected 24 h after a single dermal dose of 400 mg/kg of DEET in rats. Each par is a means \pm SE of five samples.

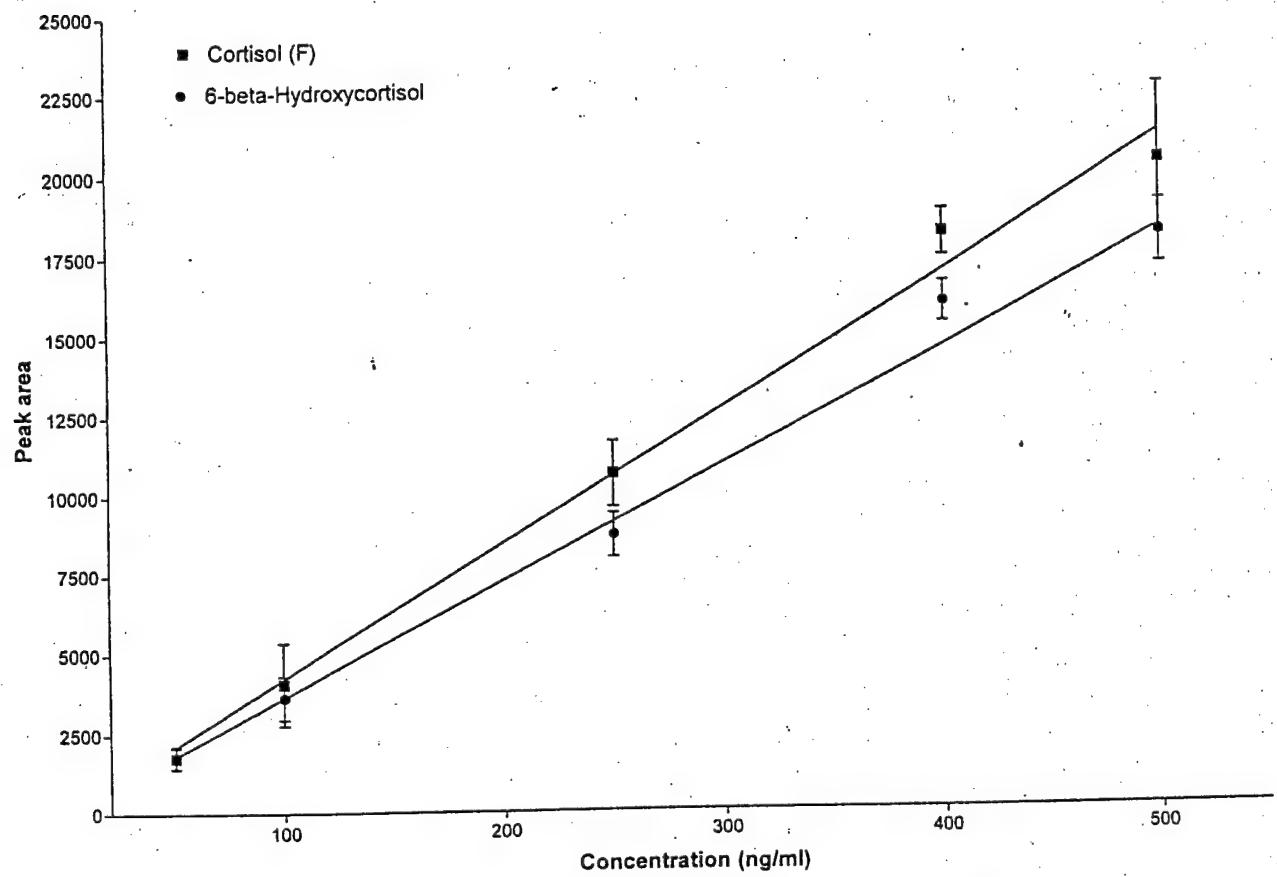


Figure 1

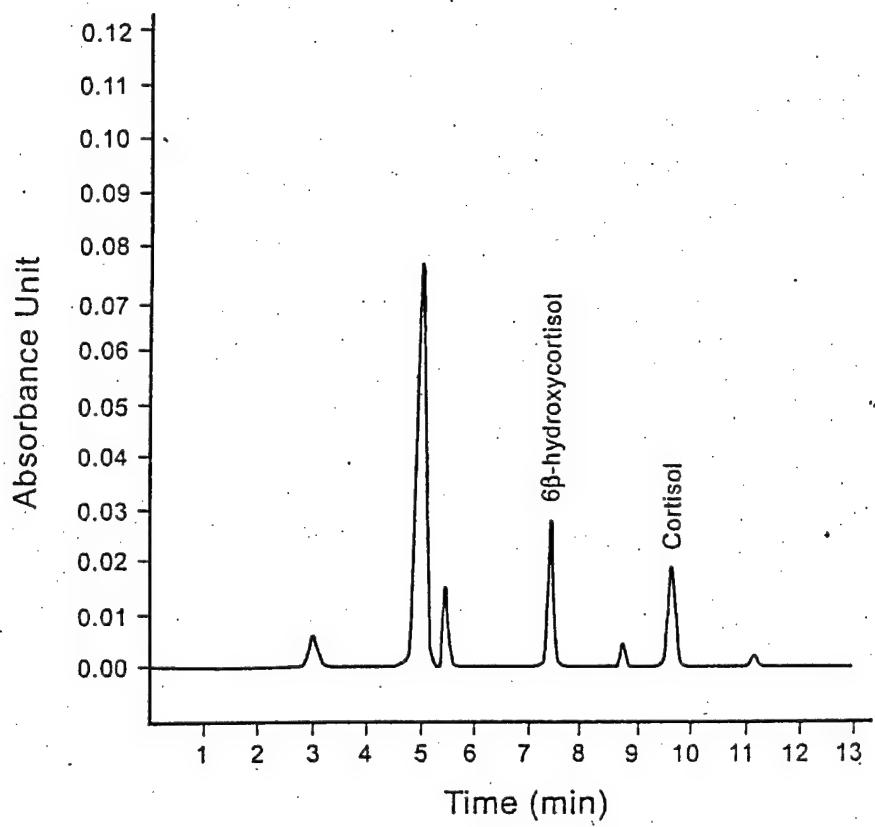


Figure 2

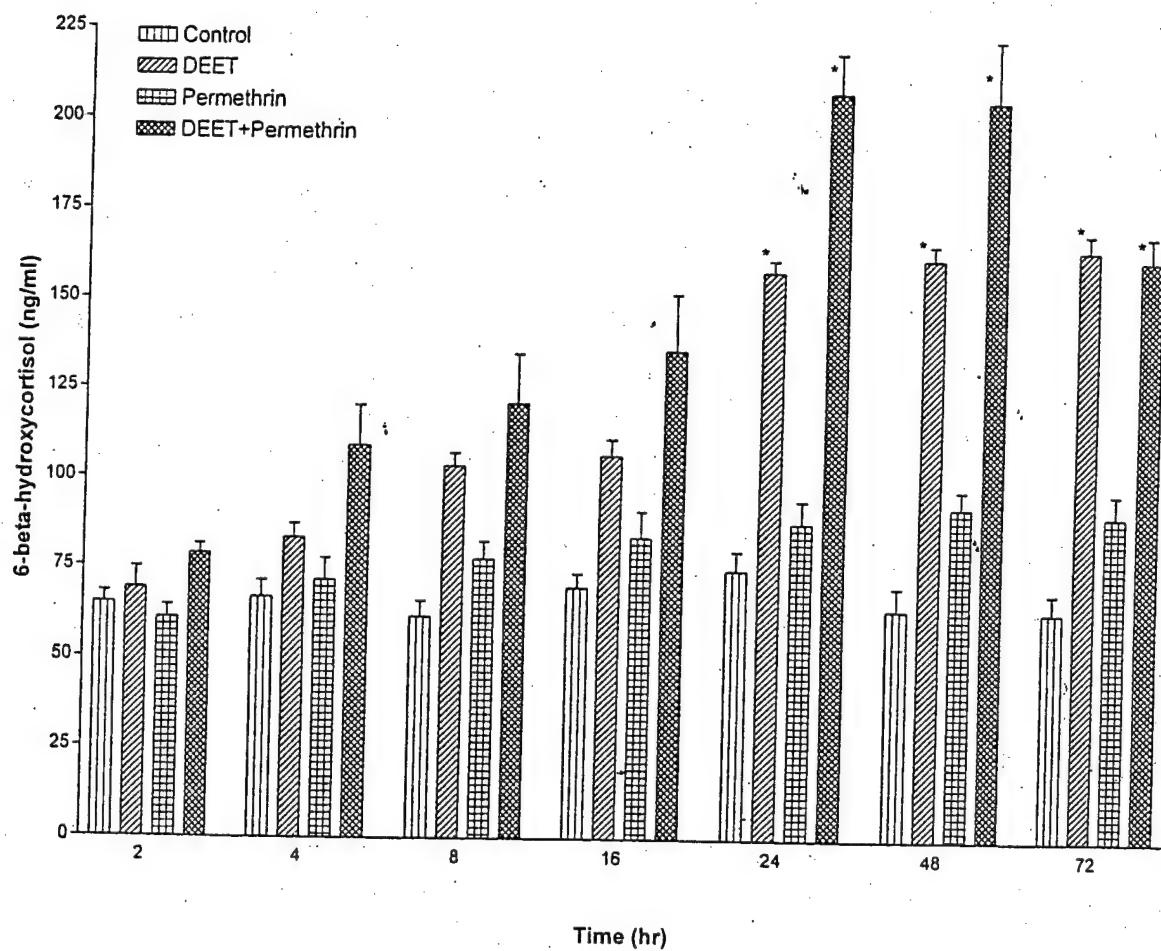


Figure 3

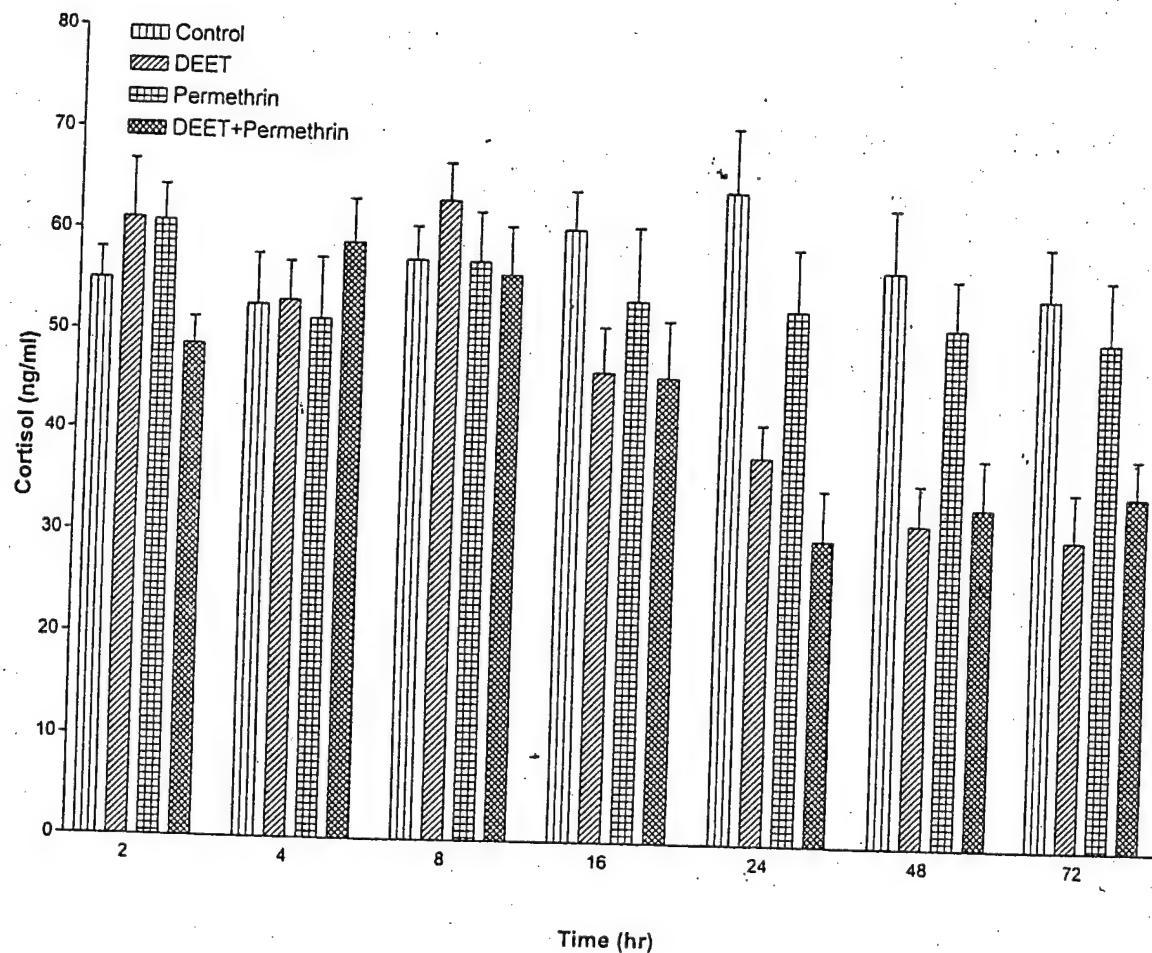


Figure 4

5. PHARMACOKINETIC INTERACTIONS BETWEEN DEET (N,N-DIETHYL-*m*-TOLUAMIDE) AND PERMETHRIN FOLLOWING DERMAL ADMINISTRATION IN RATS

Abstract

The pharmacokinetic interactions between a single dermal and intravenous (*iv*) dose of the insecticide permethrin (3-(2,2-dichloroethyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl) methyl ester) and the insect repellent DEET (*N,N*-diethyl-*m*-toluamide) has been investigated in rats. Rats were dosed dermal and intravenous with either 1.3 mg/kg (0.325 mg/1 cm² of skin area) of permethrin, and a dermal dose of 400 mg/kg (100 mg/ 1cm² of skin area) or an *iv* dose of 40 mg/kg of DEET, alone or in combination. Five rats dosed dermal were sacrificed at each time interval of 0.5, 1, 2, 4, 8, 24, 48, and 72 h after dosing. Plasma, liver, kidney, brain, testes, and urine collected and analyzed for permethrin, DEET, and their metabolites by high performance liquid chromatography (HPLC). In rats treated with a single agent, 0.5 and 72 h after dosing, the application site retained 45%, and 0.05% of DEET, and 62% and 4% of permethrin, respectively. Rate of distribution of permethrin in tissues was slower compared to DEET. At 8 hr after dosing, maximum concentrations of permethrin were 76 and 185 ng/g in liver and kidney, respectively. Permethrin metabolites *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were identified in plasma, liver and kidney 24 h after administration. DEET and its metabolites *m*-toluamide and *m*-toluic acid were detected in plasma and tissues within 1 h of dosing. The time concentration curves of DEET and permethrin in plasma following dermal or *iv* dose were fitted to a one compartment pharmacokinetic model with a terminal half-life of elimination of 18.4, 32.6 h and 11.7, 22.9 h for DEET and permethrin, following *iv* and dermal dose, respectively. DEET and

its metabolites *m*-toluamide and *m*-toluic acid were detected in urine samples before hydrolysis. Sequential enzymatic hydrolysis of urine samples showed that *m*-toluamyl glucuronide and *m*-toluamyl sulfate conjugates were excreted in urine. Neither permethrin nor its metabolites *m*-phenoxybenzoic acid and *m*-phenoxybenzyl alcohol were detected in urine samples. Sequential enzymatic hydrolysis of urine samples yielded *m*-phenoxybenzyl alcohol, indicating that *m*-phenoxybenzyl glucuronide and *m*-phenoxybenzyl sulfate were excreted in urine following permethrin administration. Hot acid hydrolysis of urine samples yielded unidentified metabolites of DEET and permethrin.

Combined administration of both compounds either *iv* or dermal significantly increased AUC_{plasma} of DEET compared to AUC_{plasma} of DEET when applied alone. Permethrin significantly increased DEET half-life of elimination from plasma following combined *iv* administration. Concurrent *iv* administration of both compounds significantly increased permethrin AUC_{plasma} . But there was no significant effect on AUC_{plasma} of permethrin in the presence of DEET following dermal administration. There was no significant effect on absorption of either compound following dermal application of their combination compared to individual application. A significant decrease in the concentration of urinary conjugated metabolites of DEET following dermal combined application of both agents has been detected. The results showed that combined exposure to permethrin and DEET could prolong presence of DEET in the circulation system, reduce its rate of elimination and levels of conjugated metabolites.

Introduction

Permethrin is a pyrethroid insecticide effective against mites and head lice (Burgess et al., 1992; Fraser, 1994; Miller, 1989). DEET is a widely used insect repellent used against mosquitoes and other biting insects (USEPA, 1998; Robinson and Cherniak, 1986; Brown and Hebert, 1997). Both compounds were used during the Gulf War for pest control (Young and Evans, 1998). Permethrin modifies sodium channel to open longer during a depolarization pulse (Narahashi, 1985). Previous studies showed that DEET has direct effect on the nervous system in laboratory animals resulting in spongiform myelinopathy in the brain stem with signs including ataxia, seizures, coma, and death (Verschoyle et al, 1990). Furthermore, extensive and repeated topical application of DEET resulted in human poisoning including two deaths (Edwards and Johnson, 1987; Roland et al., 1985). Combined exposure to permethrin and DEET enhanced neurotoxicity in hens (Abou-Donia et al, 1996), modified the blood brain barrier in rats (Abou-Donia et al, 2001a, 2001b), caused oxidative stress in rats (Abu-Qare and Abou-Donia, 2000b, 2001b, 2001c; Abu-Qare et al, 2001), increased mortality in rats (McCain et al, 1998), and caused behavioral alterations in male rats (Abou-Donia et al., 2001a; Hoy et al, 2000a, 2000b). Published reports implicated exposure to permethrin and DEET in Gulf War illnesses (Abou-Donia et al., 1996; Haley and Kurt, 1997; Olsan et al., 1998; Kurt, 1998; Shen, 1998; Wilson et al., 1998; Jamal, 1998; Haley et al., 1999). Metabolism of permethrin has been examined following oral or *i.v* dose in rats (Abu-Qare and Abou-Donia, 2000a; Anadon et al, 1991). Disposition and metabolism of DEET has been studied *in vivo* following dermal application to human volunteers (Abu-Qare and Abou-Donia, 2000a; 2001c; Seliem et al., 1995; Blomquist

and Thorsell, 1977), in rats (Schoeing et al., 1996); in mice (Blomquist and Thorsell, 1977), in cattle (Taylor et al., 1994) and after topical application in beagle dogs (Qiu et al., 1997). DEET metabolism involved *N*-dealkylation, ring hydroxylation and ring dealkylation following *in vitro* incubation with rat liver microsomes (Constantino and Iley, 1999; Taylor, 1986).

We hypothesized that combined exposure to DEET and permethrin could be resulted in enhanced toxicity. A possible pathway of interactions between these compounds is affect on metabolism and pharmacokinetic of the other. This study reports on results of absorption, disposition, metabolism and excretion of single dermal doses of permethrin, or DEET, alone and in combination in rats.

Materials and methods

Chemicals

Permethrin (99%, (3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester) was purchased from Chem Service Inc. (West Chester, PA). *m*-Phenoxybenzyl alcohol, *m*-phenoxybenzoic acid, β -glucuronidase from bovine liver type B-1, and sulfatase from *limpets* type V were obtained from Sigma Chemical Co., (St. Louis, MO). DEET ($\geq 97\%$ *N,N*-diethyl-*m*-toluamide) was obtained from Aldrich Chemical Co, Inc. (Milwaukee, WI). *m*-Toluamide, and *m*-toluic acid were purchased from Fisher Scientific (Pittsburgh, PA). C₁₈ Sep-Pak^R cartridges were obtained from Waters Corporation (Waters Corporation, Milford, MA). Water and acetonitrile (HPLC grade) were obtained from Mallinckrodt Baker, Inc. (Paris, KY).

Chromatographic conditions

The liquid chromatographic system (Waters 2690 Separation Module), consisted of a Waters 600E multisolvent delivery system pumps, a Waters Ultra WISP 715 autoinjector and a Waters 2487 dual λ absorbance detector (Waters Corporation, Milford, MA). A guard column (Supelco, 2 cm \times 4.0 mm, 5 μ m (Supelco Park, Bellefonte, PA), and a reversed-phase C₁₈ column μ Bondapak C₁₈ 10 μ m, 3.9 \times 300 mm were used, (Waters Corporation, Milford, MA) were used. The mobile phase was water (adjusted to pH 3.0 using 0.1N acetic acid): acetonitrile gradient at flow rate ranging between 1.0-1.7 ml/min. The gradient started at 1% acetonitrile, increased to 25% acetonitrile at 3.6 min, then to 45% acetonitrile at 6 min, and up to 85% acetonitrile at 11 min. The system returned to 1% acetonitrile at 14 min and was kept under these conditions for 3 min to re-equilibrate. Total run time was 17 min. The analytes were monitored by UV detection at 210 nm for permethrin, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid, and 230 nm for DEET, *m*-toluamide, and *m*-toluic acid. The chromatographic analysis was performed at ambient temperature (Figure 1).

Calibration procedures, detection limits and recoveries

Five different calibration standards of a mixture of permethrin, DEET, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were prepared in acetonitrile. Their concentrations ranged from 100-1000 ng/ml following a method developed and validated in our laboratory (Abu-Qare and Abou-Donia, 2000a). Linear calibration curves were obtained by plotting the peak areas of the individual chemicals as a function of concentration. The standard curves were used to determine recovery of the chemicals from plasma and tissue samples. Limits of detection were determined at the

lowest concentration that can be detected, taking into consideration a 1:3 baseline noise: calibration point ratio. Plasma, urine and tissue samples from control animals were spiked with selected concentration ranging between 100-1000 ng/ml of each permethrin, DEET and metabolites (Abu-Qare and Abou-Donia, 2000a). The samples were extracted and cleaned up as described under sample preparation. Percentage recoveries of each compound were determined using the calibration curves as described above.

Animals treatment and handling

Sprague Dawley rats (250-300 g) were purchased from Zivic Miller (Zelienople, PA). The animals were kept in a 12-h light/dark cycle (temperature 21-23°C) and were provided with a free supply of feed (Rodent Laboratory Chow, Ralston Purine Co., (St. Louis, MO) as well as tap water. Animal care was conducted according to institutional guidelines. Permethrin and DEET were dissolved in 70% ethanol. A single dose of 1.3 mg/kg of permethrin or 400 mg/kg of DEET, or both doses in combination (DEET followed by permethrin) were applied with a micropipette (1 ml/kg) to an unprotected 1 cm² area of pre-clipped skin on the back of each rat. The application area was not protected to resemble real-life situations. A group of five animals was used for each time point. The doses were selected to represent real-life exposure levels (Abou-Donia et al., 1996; 2001a. 2001b). Five control animals were treated with equal volume of 70% ethanol either to resemble single application (250 µl) or combined application (500 µl) and kept under similar conditions as treated rats. Rats were placed into metabolic cages after dosing to facilitate urine collection and five rats per time point were anesthetized with halothane (Halothane Laboratories, River Edge, PA) at intervals of 0.5,

1, 2, 4, 8, 24, 48, and 72 hr after dosing and exsanguinated by cardiac puncture into heparinized syringes. Samples of liver, kidney, brain and testes were collected from each animal and portion of the blood volumes were separated into plasma samples by centrifugation at 2400 rpm for 10 min at 5°C. Plasma and tissue samples were stored at -70°C until analysis.

Sample preparation

Plasma (0.5 ml), and urine (1.0 ml) sample, or 0.5 g of liver, kidney, brain, and testes were each mixed or homogenized with a 2 ml of (1:1) of acetonitrile and methanol, centrifuged at 1000g for 5 min, and the supernatant was removed. Disposable C₁₈ Sep-Pak Vac 3cc (500mg) cartridges were conditioned with 3 ml of acetonitrile, then equilibrated using 3 ml of water prior to use. The supernatant was loaded into the disposable cartridges, washed by 3.0 ml of water, followed by elution with 2 ml of methanol, and 2 ml of acetonitrile. The elution volume was reduced to 500 µl (0.5 ml) in a test tube rack using a gentle stream of nitrogen, prior to analysis by HPLC.

Urine Analysis

Non-conjugates

A portion of each urine sample (1 ml) was acidified using 0.1N phosphoric acid (pH 4) and passed through Sep pack cartridges as described above. The contents were analyzed using HPLC to determine parent compound and non-conjugated metabolites. Another portion of the same urine sample (1ml) was used for determination of the conjugates as describe below.

Conjugates

(a) Enzymatic hydrolysis

- i) Glucuronides. β -glucuronidase from bovine liver type B-1 was added to urine residues in 0.2 M sodium acetate buffer (pH 4.5), incubated for 18 hours at 37°C, then the urine incubate was analyzed as previously described under sample preparation,
- ii) Sulfates. The remaining urine residue was incubated for 24 hours at 37°C with sulfatase from *limpets* type V in 0.2 M sodium acetate buffer (pH 4.5). The residue was analyzed as described above under sample preparation.

(b) Hot acid hydrolysis

Another 1.0 ml urine sample was adjusted by 1 N sulfuric acid to (pH 2.5), heated for 2 hours at 80°C, and analyzed as described above.

Kinetics analysis

The kinetic analysis of permethrin, DEET and metabolites in plasma and tissues were performed using WinNonlin Program (Pharsight Corporation, Mountain View, CA). The terminal half-life of permethrin, DEET and their metabolites was calculated from the elimination rate constant K_{10} , that was obtained by linear regression of the terminal linear exponential decline in concentration. The total area under the compound concentration *vs.* time curves for plasma, AUC_{plasma} , or brain AUC_{brain} was calculated by the trapezoidal rule and extrapolated to infinity by using the last data point and the respective terminal linear experimental decline.

Statistical analysis

The pharmacokinetic parameters were subjected to analysis of variance (ANOVA) to determine if the difference between treatment with the individual compound and the combination is significance using a GraphPad Prism program for Windows (GraphPad Software, Inc., San Diego, CA). A value of $P<0.05$ was considered statistically significant.

RESULTS

Clinical observation

A single dermal dose of 1.3 mg/kg of permethrin, or a single dermal dose of 400 mg/kg of DEET, or both agents at these doses in combination did not produce observable toxic effect in rats, based on gross examination without morphological or physiological studies carried out. Both control and treated animals consumed comparable amounts of feed and water. There was no difference in weight, size, shape, or color of various tissues of treated animals compared to tissues of control rats.

Limits of detection and recovery of permethrin, DEET, and metabolites

Average percentage recoveries of permethrin, DEET, *m*-toluamide, *m*-toluic acid, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid from plasma were 82.3 ± 8.2 , 71.1 ± 11.2 , 73.6 ± 10.1 , 79.3 ± 8.5 , 84.3 ± 12.0 , and 82.6 ± 6.1 , respectively. Their limits of detection were 30, 50, 50, 80, 20, and 30 ng/ml, respectively.

Disappearance of permethrin and DEET from the application site

Permethrin and DEET were disappeared at different rate from rat skin following a single dermal dose. The application site retained 62% and 4% of permethrin, and 45% and 0.05% of DEET after 0.5 hr and 72 hr following application, respectively. Rate of disappearance of both compounds was more rapid during the early time after application, then gradually decreased (Table 1)

DEET and permethrin in plasma and tissues

Tables 2 and 3 list the concentrations of permethrin and DEET (ng /g fresh tissue or mg/ ml plasma) at different time intervals following a single dermal dose of both chemicals in rats. Permethrin, DEET and their metabolites were distributed and detected in analyzed tissues. Permethrin was found in the kidney, liver, brain and plasma. Maximum concentration of DEET was also detected in the analyzed tissues in the following order kidney > liver > plasma > brain > testes. Following administration of a combined dose of permethrin and DEET, higher amount of DEET was detected in plasma (Table 4), while there was no significant change in the pattern of distribution and amount of permethrin and its metabolites when it was concurrently applied with DEET (Table 5).

Metabolism of permethrin and DEET

Metabolites of permethrin and DEET were analyzed using high performance liquid chromatography (HPLC) (Fig 1). *m*-Phenoxybenzyl alcohol, a metabolite of permethrin, was detected in liver, while *m*-phenoxybenzoic acid was detected in liver, kidney and plasma over the time course of analysis. DEET metabolites *m*-toluic acid and

m-toluamide were found in all analyzed tissues and plasma (Tables 2 and 3). Proposed metabolic pathways of permethrin and DEET are shown in Figures 2 and 3.

Urinary excretion of DEET and permethrin following dermal dose

Urinary excretion was rapid following dermal application of DEET. DEET and its metabolites *m*-toluamide and *m*-toluic acid were identified in urine shortly after application. Following enzymatic hydrolysis with glucuronidase, level of DEET metabolites increased, indicating that *m*-toluamyl and toluyl glucuronide conjugates were excreted in the urine (Table 6). Incubations of urine samples with sulfatase resulted in a significant increase in the concentrations of both metabolites, a further indication of existence of *m*-toluamyl sulfate and *m*-tolyl sulfate conjugates (Table 6). Urinary excretion of permethrin metabolites was slower compared to DEET. A similar trend was detected following incubation of permethrin urinary excretion with glucuronidase and sulfatase. *m*-Phenoxybenzyl alcohol was detected (Table 7). Acid hydrolysis of urine samples produced unidentified metabolites for both DEET and permethrin.

Bioavailability of DEET and permethrin.

Plasma concentrations of DEET, permethrin and their metabolites following *iv* dose are shown in tables 8 and 9. The systemic bioavailability of DEET was 45% and 40% when administered alone and in combination with permethrin, respectively (Table 10). Permethrin bioavailability was 22% and 16% when applied alone or in combination with DEET, respectively (Table 11).

Pharmacokinetics profiles of permethrin, DEET.

Permethrin and DEET disappeared mono and bi-exponentially from plasma following a single dermal and *iv* dose in rats, respectively. The time concentration curves of permethrin and DEET in plasma were fitted to one and two compartment model, respectively. The pharmacokinetic parameters of permethrin, DEET are listed in Table 10 and 11.

Pharmacokinetic interactions

Concurrent administration of permethrin and DEET had no significant effect on rate of disappearance of either compound from the site of application (Table 1). Pharmacokinetic profiles of permethrin, and DEET following *iv* or dermal combined administration revealed that permethrin significantly increased DEET AUC_{plasma} (Figures 4 and 6). Also an *iv* dose of permethrin significantly increased DEET half-life of elimination from the plasma (Table 10). DEET significantly increased the AUC_{plasma} of permethrin following *iv* concurrent administration (Figure 5). But there was no significant effect on permethrin pharmacokinetic profiles following dermal concurrent application with DEET (Figure 7). The concurrent application of both compounds decreased rate and levels of DEET urinary excretion (Figure 8).

Discussion

A single dermal dose of 400 mg/kg of DEET was rapidly disappeared from the application site and distributed in tissues after administration in rats. These results are in agreement with previous reports on absorption and disposition of DEET in rats (Schoenig

et al., 1996), in mice (Blomquist and Thorsell, 1977), in beagle dogs (Qiu et al., 1997), and following dermal application to human volunteers (Seliem et al., 1995). Furthermore, DEET has been shown to rapidly cross the dermal barrier following topical application in rats (Windhenser et al., 1982). In the present study DEET was applied in ethanol. Its pattern of absorption could be different if applied in other carrier vehicles. Qiu et al. (1998) reported that *in vitro* skin permeation, the vehicle showed marked effect on rat skin penetration of DEET. Absorption of DEET was significantly correlated to the type of commercial formulation following application in rats (Stinecipher and Shah, 1997). Our findings showed that while 11% of the applied dose of DEET was retained by the application site 24 h after application, more than 99% of the applied dose of DEET disappeared from the application site 72 h after application. These data are consistent with those reported by Schoeing (1996), that 16% of the dose was retained by the application site 36 hr after dermal dose of DEET in human volunteers and by Taylor et al. (1994) that 72.9% of DEET was absorbed into the systemic circulation following dermal single dose in cattle.

DEET was distributed rapidly in the tissues analyzed with a maximum concentration found within 4-8 hr after application; liver>kidney>plasma>brain>testes. In previous study, Blomquist and Thorsell (1977) reported that blood, kidney and liver had high concentration of radioactivity after cutaneous application of ^{14}C -*N,N*-diethyl-*m*-toluamide in mice.

Disappearance of permethrin from the application site was slower compared to DEET, possibly due to differences between the compounds in their molecular weights and physical properties such as lipid solubility. Only 4% of administered dose of permethrin

was retained by the application site after 72 h. In addition to its absorption by rat skin, permethrin and DEET could be dissipated from rat skin through other pathways such as volatilization, or through contact with surfaces of metabolic cages.

Permethrin and metabolites were detected in tissues and plasma within 0.5-24 hr after application. The findings are consistent with the results of Anadon et al (1991) who reported that permethrin was distributed in tissues and accumulated in the nervous system following an oral dose of 460 mg/kg in rats.

Figures 2 and 3 present a suggested scheme for the metabolism of dermal dose of DEET and permethrin. DEET metabolites *m*-toluamide and *m*-toluic acid were detected in some of the analyzed tissues. In this study, DEET underwent metabolic transformation mediated by esterases and cytochrome p450 enzymes, resulted in formation of metabolites of *m*-toluic acid and *m*-toluamide. This is in agreement with previous reports following dermal dose of DEET in rats and dogs (Schoenig et al, 1996; Qiu et al, 1997), and in accordance with previous studies reported that DEET metabolism mediated by *N*-dealkylation, ring hydroxylation and ring dealkylation following *in vitro* incubation with rat liver microsomes (Constantino and Iley, 1999; Taylor, 1986). These findings are also consistent with our recent results obtained following *in vitro* incubation of DEET with human liver microsomes (Abu-Qare and Abou-Donia, 2001d).

In this study, DEET metabolite *m*-toluic acid was detected in plasma 1 hr after application indicates the rapid hydrolysis of DEET *in vivo*. Permethrin and its metabolites *m*-phenoxybenzyl alcohol and *m*-phenoxybenzoic acid were also detected at 2 h after application in plasma and tissues, showing the role of esterases in its metabolism. In previous results, permethrin metabolites *m*-phenoxybenzyl alcohol, and

m-phenoxybenzoic acid were detected in plasma and tissues up to 48 h after an oral dose in rats (Anadon et al, 1991). Metabolism of permethrin reported in this study indicates its hydrolytic cleavage as an initial phase of metabolism. Rapid hydrolysis of permethrin was catalyzed by butyrylcholinesterases following *in vitro* incubation with human plasma, where addition of butyrylcholinesterase inhibitor Iso-OMPA significantly inhibited permethrin metabolism *in vitro* (Abu-Qare and Abou-Donia, 2001d). Furthermore, permethrin metabolism proceeded through oxidation and the formation of *m*-phenoxybenzoic acid. This is in accordance with a report that permethrin oxidative pathway is mediated by cytochrome P450 enzymes, thus more toxicity has been detected when an oxidase inhibitor PB (piperonyl butoxide) was applied with permethrin in rats (Vulule et al, 1999). In other study, permethrin induced cytochrome P-4502B in rat liver (Koska et al, 1997).

The present results suggest that the pharmacokinetics analysis of DEET following a single dermal dose of 400 mg/kg would require a one-compartment open-model system. Elimination half-life of DEET was 32 h, while T_{max} in plasma was 2.1 h and C_{max} was 2103 ng/ml. In their study, Hoy et al. (2000) reported that blood serum concentration of DEET was 12347 ng/ml following an i.p injection of 500 mg/kg in male rats. In another study, DEET's elimination half-life was 200.2 min following a dermal dose in human volunteers (Schoeing, 1996). Following its administration, only small amount of the intact DEET was excreted in the urine. Majority of the urinary excretion following a single dermal dose of DEET and permethrin was in the form of conjugated metabolites. This was confirmed following sequential enzymatic hydrolysis with glucuronidase and sulfatase.

The results also suggest that permethrin is also fitted to one compartment pharmacokinetic model, with half-life of elimination from plasma of 22.9 h. This is in accordance with Anadon et al (1991) who suggested one compartment model for the plasma profile of permethrin following oral dose of 460 mg/kg or *i.v* dose of 46 mg/kg in rats, where elimination half-life of permethrin was from rat plasma following an oral dose was 12.37 hr.

The results show that dermal administration of a combined dose of DEET and permethrin resulted in a significant increase in the AUC_{plasma} of DEET in rats, with a significant increase in its half-life of elimination following *iv* dose, and a decreased rate of its urinary excretion. In contrast to the effect of permethrin on DEET in rat plasma, DEET did not significantly affect permethrin kinetics after dermal administration. This is could be due to the low dose of permethrin used compared to DEET, and to a rapid hydrolytic cleavage of permethrin, presumably by esterases. This trend of rapid cleavage of permethrin has been reported *in vivo* and *in vitro* incubation with human plasma (Abu-Qare and Abou-Donia, 2000a; Abu-Qare and Abou-Donia, 2001d). Moss (1996) also reported that DEET inhibited hydrolysis of some but not all of cholinesterase inhibitors in German cockroaches.

In this study, DEET and permethrin were applied in 70% ethanol, and at different application sites on the back of the neck of rats. This was to avoid possible interaction at the application site. The findings suggest there was no significant effect of each compound on absorption of the other. Our findings of *in vivo* disappearance of Coadministered DEET and permethrin do not agree with previous *in vitro* studies in which DEET was administered concomitantly with another insecticide or drug. When

both DEET and permethrin were coadministered to rodent and pig skin *in vitro*, DEET significantly decreased permethrin absorption (Baynes et al., 1997), and significantly enhanced persistence of the pyrethroide insecticide fenitrothion by rat skin *in vitro* (Moody et al, 1987). Furthermore, addition of DEET to the gel of the drug methotrexate resulted in two-fold increased permeation of methotrexate into muscle within 4 h after dosing in rabbits (Lu et al., 1997). The gradual decrease in rate of disappearance of DEET and permethrin starting 24 h after application could be due to the saturation of the application site at that time, or to their binding to skin constituents.

In summary, co-administration of permethrin and DEET increased DEET concentration in the plasma, decreased its urinary excretion, and enhanced its persistence in the body as evident from AUC_{plasma} following combined application. This effect may be due to a competition between permethrin and DEET on certain metabolic pathway, thus reducing the DEET rate of metabolism. In a recent study in our laboratory, permethrin inhibited DEET dissipation following *in vitro* incubation by human liver microsomes (Abu-Qare and Abou-Donia, 2001d). In a previous study by Hoy et al. (2000a), cutaneous administration of 15 mg/kg of permethrin with 5 mg/kg of pyridostigmine bromide increased permethrin concentration in serum of male and female rats compared to permethrin when applied alone at rate of 30 mg/kg.

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Figure legends

Figure 1: HPLC profile of DEET, permethrin and their metabolites A) *m*-toulamide, B) *m*-toluic acid, C) DEET, D) *m*-phenoxybenzyl alcohol, E) *m*-phenoxybenzoic acid, and F) permethrin.

Figure 2: Suggested metabolic pathways of DEET following a single dermal dose in rats.

Figure 3: Proposed metabolic pathways of permethrin following a single dermal dose in rats.

Figure 4: Time course changes of DEET concentration in plasma of rats after a single intravenous dose, alone and in combination with permethrin.

Figure 5: Time course changes of permethrin concentration in plasma of rats after a single intravenous dose, alone and in combination with DEET .

Figure 6: Time course changes of DEET concentration in plasma of rats after a single dermal dose, alone and in combination with permethrin. .

Figure 7: Time course changes of permethrin concentration in plasma of rats after a single intravenous dose, alone and in combination with DEET.

Figure 8: Urinary excretion of DEET and metabolites after a dermal single dose, alone and in combination with permethrin.

Figure 9: Urinary excretion of permethrin metabolites after a dermal single dose, alone and in combination with DEET.

Table I Retention^a of DEET^b and permethrin by rat skin following a single dermal dose of 400 mg/kg of DEET, and 1.3 mg/kg of permethrin, alone and in combination in Sprague-Dawley rats.

Time (h)	DEET	Permethrin	DEET in the presence of permethrin	Permethrin in the presence of DEET
0.5	45.6±7.8	62.3±13.4	51.3±9.0	72.6±8.5
1	32.7±12.6	57.8±10.9	43.8±9.8	65.6±6.7
2	28.6±6.5	49.2±7.6	36.5±5.6	54.6±7.2
4	22.1±8.2	38.9±12.6	30.1±8.7	45.6±7.2
8	18.7±9.6	29.7±6.3	25.6±9.0	33.7±5.9
24	11.2±5.6	21.1±10.9	18.6±5.6	18.9±7.0
48	1.5±1.2	11.7±5.4	5.3±4.5	9.1±4.6
72	0.05±0.03	4.2±1.5	0.5±0.3	6.1±3.0

^a Results are expressed as percentage of the applied dose (mean±SD) of five animals.

^b DEET and permethrin were applied in ethanol.

Table 2 Tissue concentration^a of DEET and metabolites following a single dermal dose of 400 mg/kg in Sprague-Dawley rats.

Time (h)	DEET				<i>m</i> -Toluic acid				<i>m</i> -Toluamide			
	Plasma	Liver	Kidney	Brain	Testes	Plasma	Liver	Kidney	Plasma	Liver	Kidney	
0.5	1472±361	921±219	891±721	241±101	N.D	241±65	202±131	98±73	132±96	169±102	34±26	
1	1536±645	1204±532	956±207	247±123	N.D	313±148	119±75	112±76	245±93	301±163	101±64	
2	1792±823	1650±302	1460±372	351±112	N.D	401±230	102±56	134±82	387±128	486±121	183±58	
4	2103±631	1832±534	1609±237	260±134	N.D	391±90	187±69	174±101	460±153	512±201	290±259	
8	1776±405	2209±709	1967±452	210±108	N.D	301±113	112±64	110±32	512±234	601±187	124±23	
24	1294±320	1645±497	1830±643	198±93	181±54	109±76	N.D	125±68	460±254	245±123	N.D	
48	852±209	1034±420	1273±297	76±34	165±63	N.D	N.D	N.D	N.D	N.D	N.D	
72	276±132	354±98	675±245	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	

^a Results are expressed as ng/g fresh tissue or ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).

b DEET was dissolved in ethanol.

Table 3 Tissue concentration^a of permethrin^b and metabolites following a single dermal dose of 1.3 mg/kg in Sprague-Dawley rats.

Time (h)	Permethrin				<i>m</i> -Phenoxybenzyl alcohol				<i>m</i> -Phenoxybenzoic acid		
	Plasma	Liver	Kidney	Brain	Testes	Plasma	Liver	Kidney	Plasma	Liver	Kidney
0.5	46±13	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
1	94±16	N.D	148±42	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
2	97±41	65±27	153±29	N.D	N.D	56±12	N.D	42±19	102±13	73±8.6	N.D
4	103±37	59±31	183±101	N.D	N.D	93±31	71±23	56±13	97±9.5	82±17	N.D
8	130±29	76±35	185±92	49±32	N.D	57±10	68±41	59±20	83±16	89±26	72±9.2
24	193±54	63±12	109±63	52±27	N.D	N.D	63±19	73±16	132±52	126±40	63±13
48	112±51	N.D	73±31	N.D	N.D	N.D	78±36	68±23	121±26	123±28	78±21
72	N.D	N.D	68±29	N.D	N.D	N.D	N.D	49±21	N.D	68±13	N.D

^a Results are expressed as ng/g fresh tissue or ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).

^b Permethrin was dissolved in ethanol.

Table 4 Tissue concentration^a of DEET^b and metabolites following concurrent application of a single dermal dose of 400 mg/kg of DEET, and 1.3 mg/kg of permethrin in Sprague-Dawley rats.

Time (h)	DEET				<i>m</i> -Toluic acid				<i>m</i> -Toluamide			
	Plasma	Liver	Kidney	Brain	Testes	Plasma	Liver	Kidney	Plasma	Liver	Kidney	
0.5	1391±601	1126±349	931±218	301±97	N.D	183±76	213±138	113±29	189±18	156±53	91±63	
1	1382±362	1163±521	1073±308	273±101	N.D	291±118	224±109	146±53	269±87	297±101	176±23	
2	1613±247	1593±367	1317±418	296±114	N.D	392±78	163±78	172±47	382±156	397±162	218±49	
4	2063±214	1901±461	1729±328	243±108	N.D	461±193	149±61	162±53	496±218	523±297	293±116	
8	2416±608	2314±561	1923±216	212±93	93±12	482±213	109±45	116±35	559±296	591±213	217±63	
24	2018±538	2102±391	1971±273	172±81	216±72	135±126	87±28	N.D	362±129	263±116	81±45	
48	1046±291	967±218	1091±259	83±29	N.D	N.D	N.D	N.D	N.D	N.D	N.D	
72	358±216	193±91	416±138	N.D	N.D	N.D	N>D	N.D	N.D	N.D	N.D	

^a Results are expressed as ng/g fresh tissue or ml plasma (mean±SD) of 10 samples from five animals (two samples/animal).

^b DEET and permethrin were dissolved in ethanol.

Table 5 Tissue concentration^a of permethrin^b and metabolites following concurrent application of a single dermal dose of 1.3 mg/kg of permethrin and 400 mg/kg of DEET in Sprague-Dawley rats.

Time (h)	Permethrin				<i>m</i> -Phenoxybenzyl alcohol				<i>m</i> -Phenoxybenzoic acid		
	Plasma	Liver	Kidney	Brain	Testes	Plasma	Liver	Kidney	Plasma	Liver	Kidney
0.5	48±14	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
1	87±29	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
2	103±47	73±68	92±53	N.D	N.D	72±39	N.D	64±18	113±26	92±36	N.D
4	123±62	102±37	136±29	57±34	N.D	101±23	78±61	83±22	103±47	106±24	N.D
8	142±76	113±42	172±32	68±42	N.D	69±41	83±42	108±29	147±39	90±27	126±49
24	187±56	78±29	111±28	59±31	N.D	52±13	71±26	127±53	97±19	83±42	107±23
48	97±13	N.D	68±19	53±27	N.D	N.D	63±19	98±60	73±21	N.D	101±57
72	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D

^a Results are expressed as ng/g fresh tissue of ml plasma (mean± SD) of 10 samples from five animals (two samples/animal).

^b Permethrin and DEET were applied in ethanol.

Table.6 Pharmacokinetic parameters^a of DEET in rat plasma following a single dermal dose of 400 mg/kg, alone and in combination with 1.3 mg/kg of permethrin.

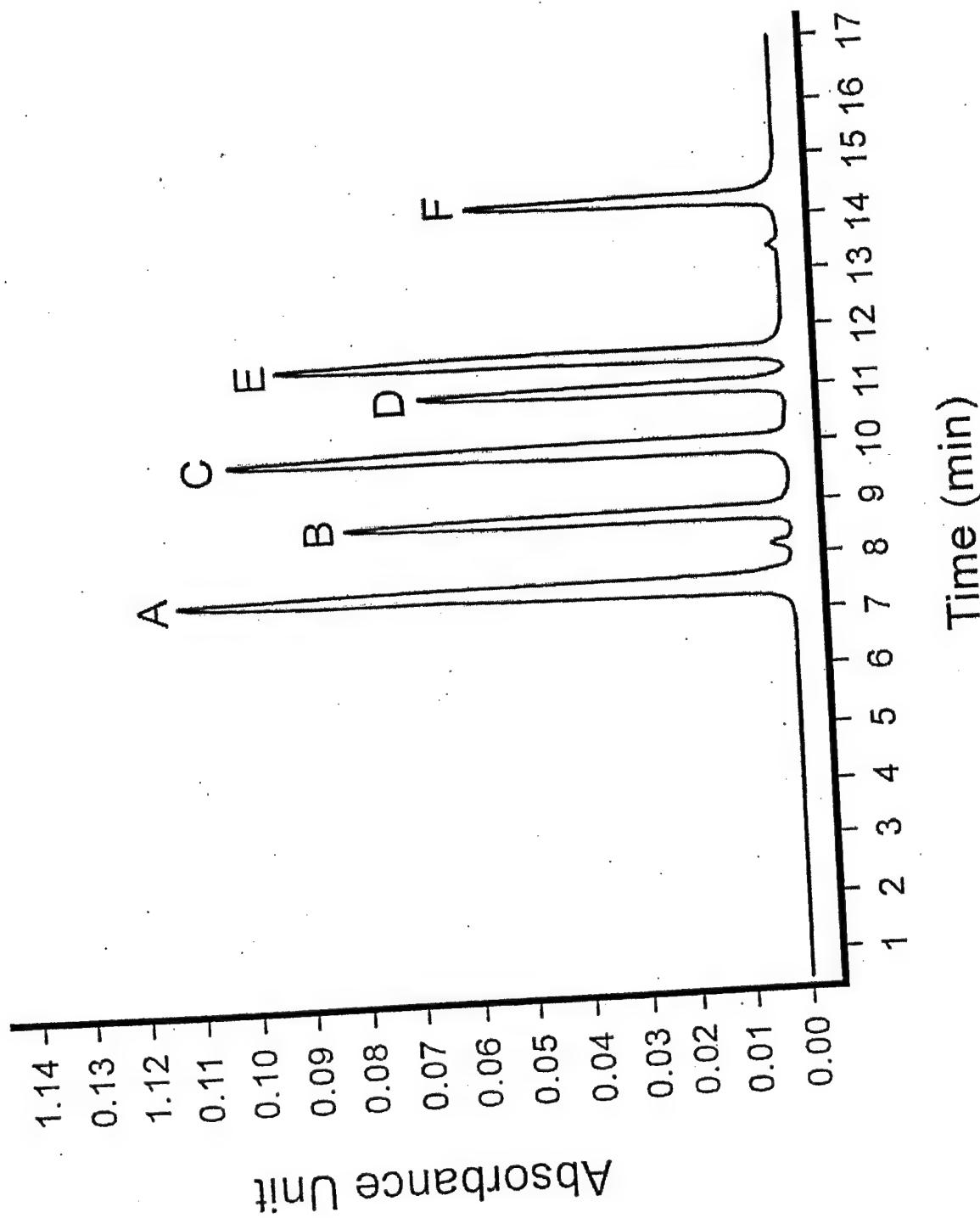
	AUC (ng.hr/ml)	C _{max} (ng/ml)	T _{max} (hr)	T _{1/2} (hr)	K ₁₀ (hr ⁻¹)
DEET alone	96985±10763	2103±631	2.3±0.5	32.6±6.3	0.02±0.004
DEET, in the presence of permethrin	133345±11703	2416±261	4.9±1.3	36.1±12.8	0.019±0.007

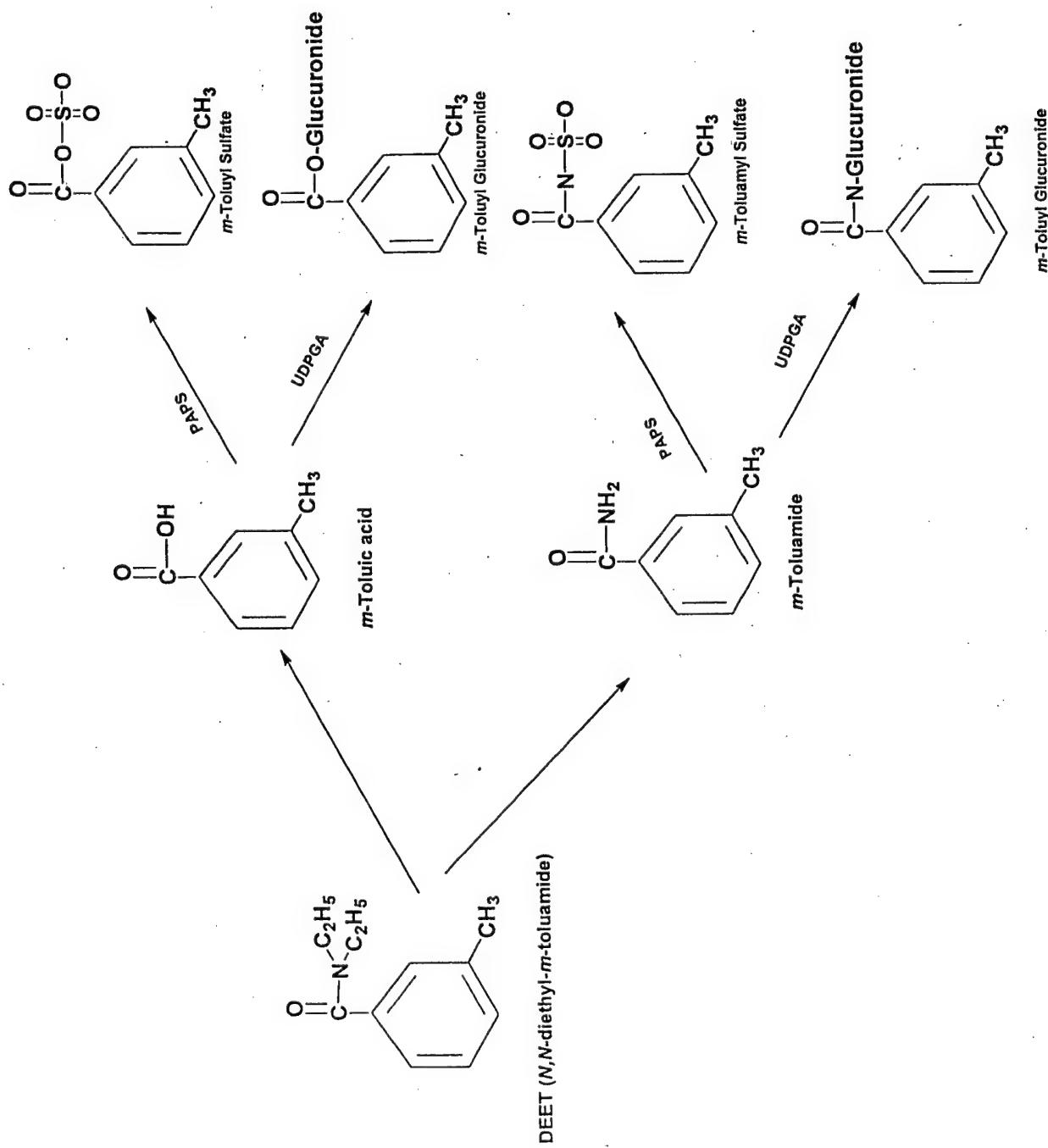
^a All parameters are defined under methods.

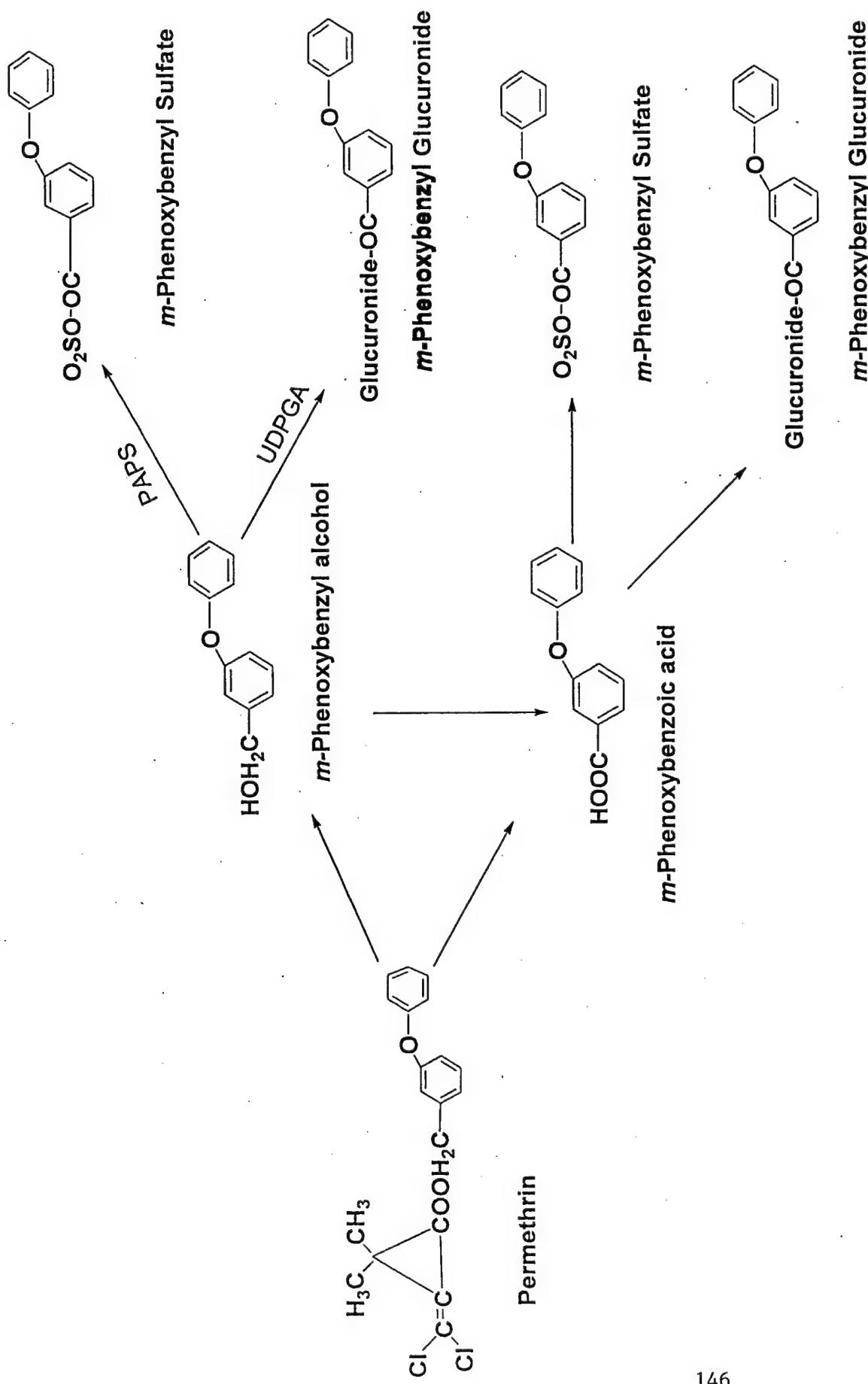
Table.7 Pharmacokinetic parameters^a of permethrin in rat plasma following a single dermal dose of 1.3 mg/kg, alone and in combination with 400 mg/kg of DEET.

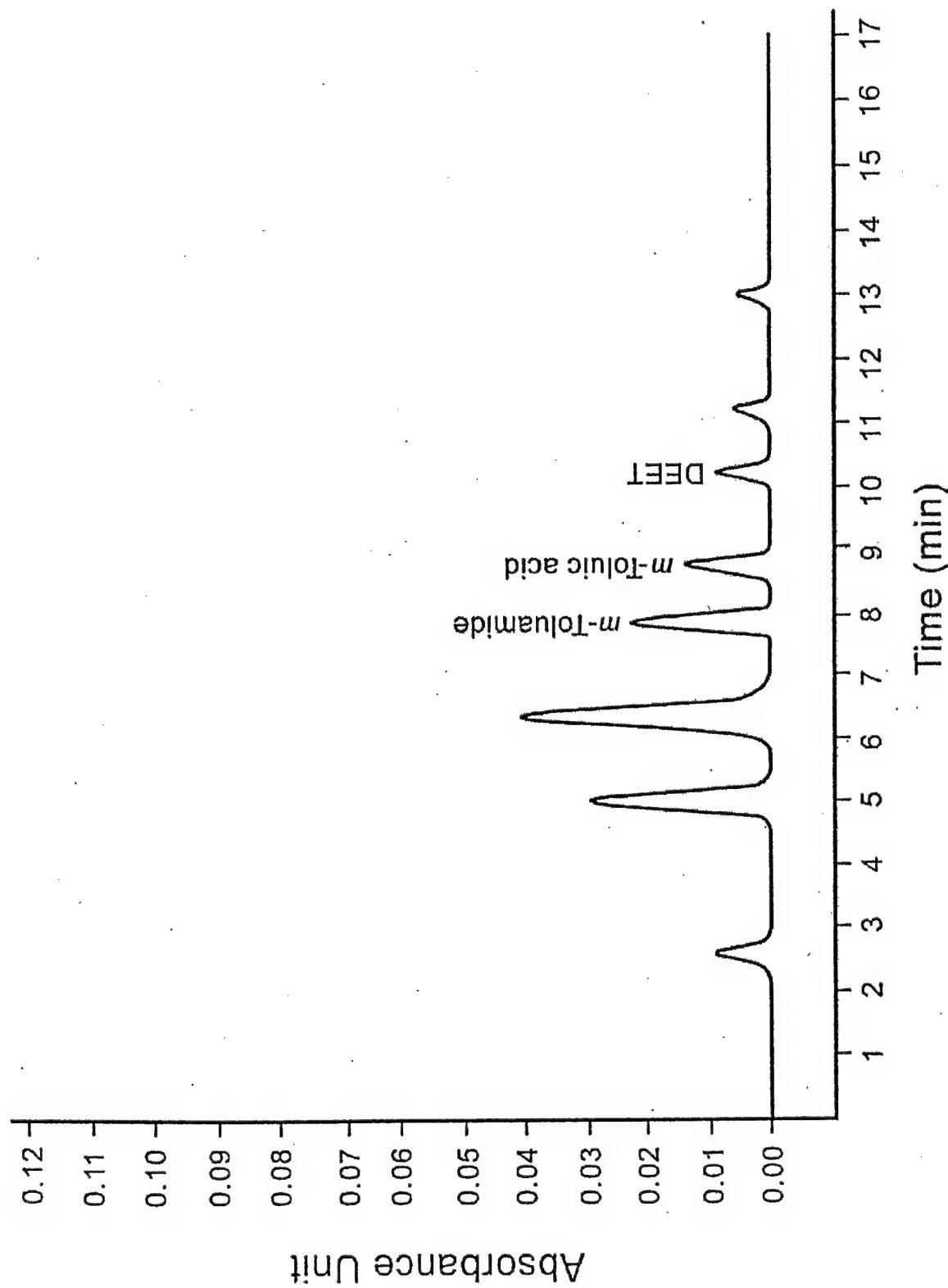
	AUC (ng.hr/ml)	C _{max} (ng/ml)	T _{max} (hr)	T _{1/2} (hr)	K ₁₀ (hr ⁻¹)
Permethrin alone	10502±2859	193.7±54	15.6±3.7	22.9±5.9	0.033±0.0026
Permethrin, in the presence of DEET	10637±2849	187.5±56	14.7±2.9	25.7±8.5	0.023±0.011

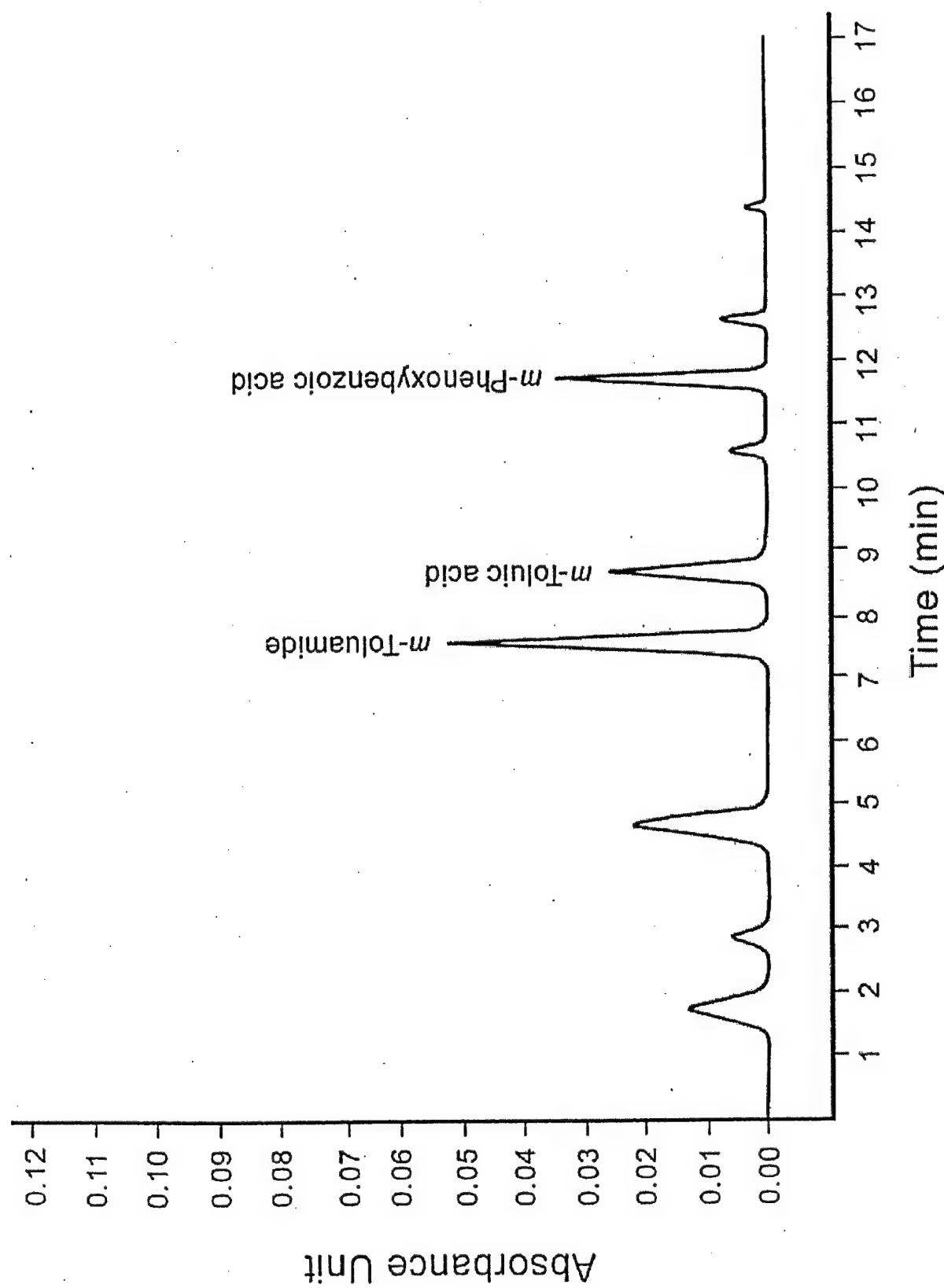
^a All parameters are defined under methods.

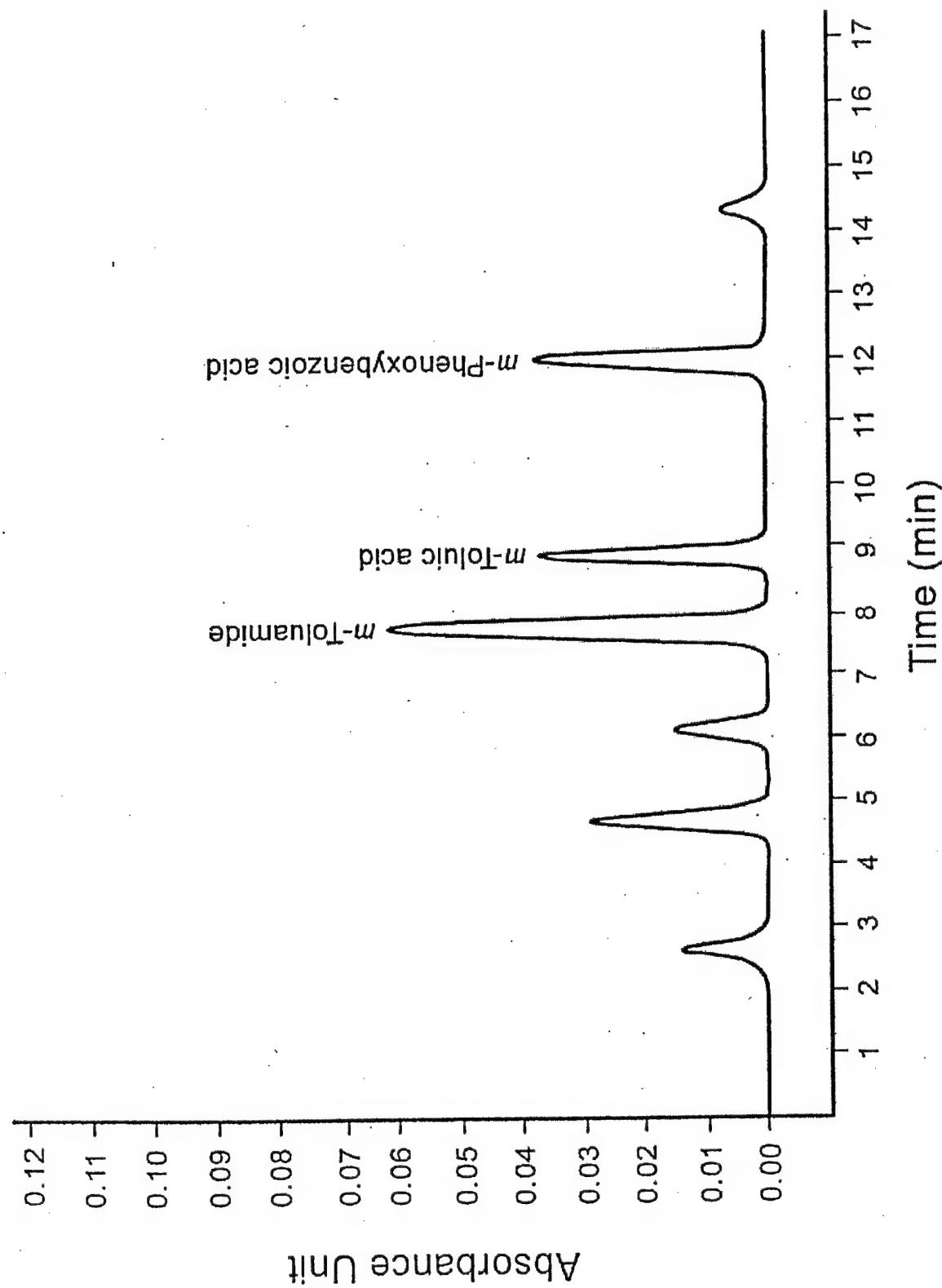


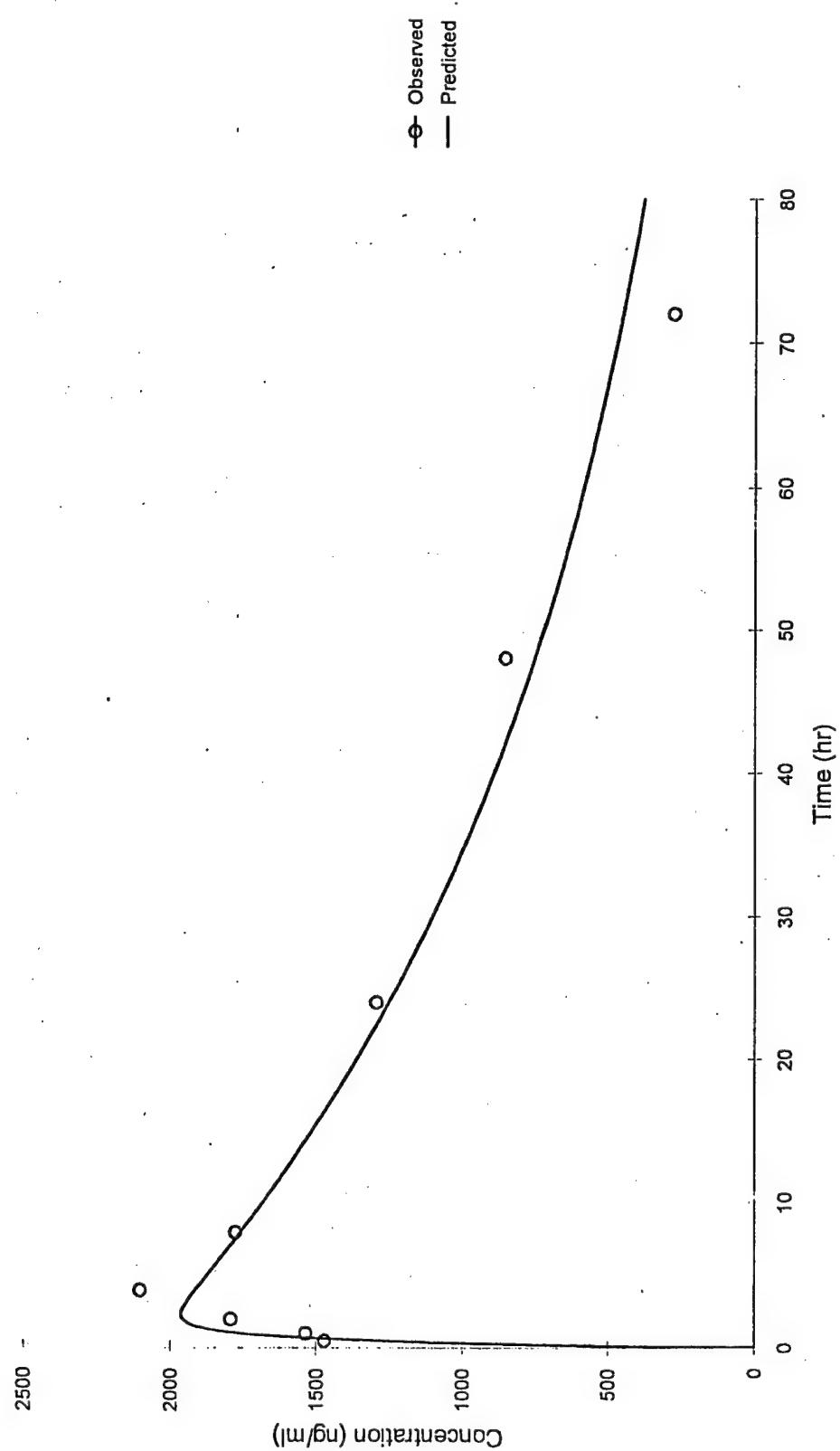


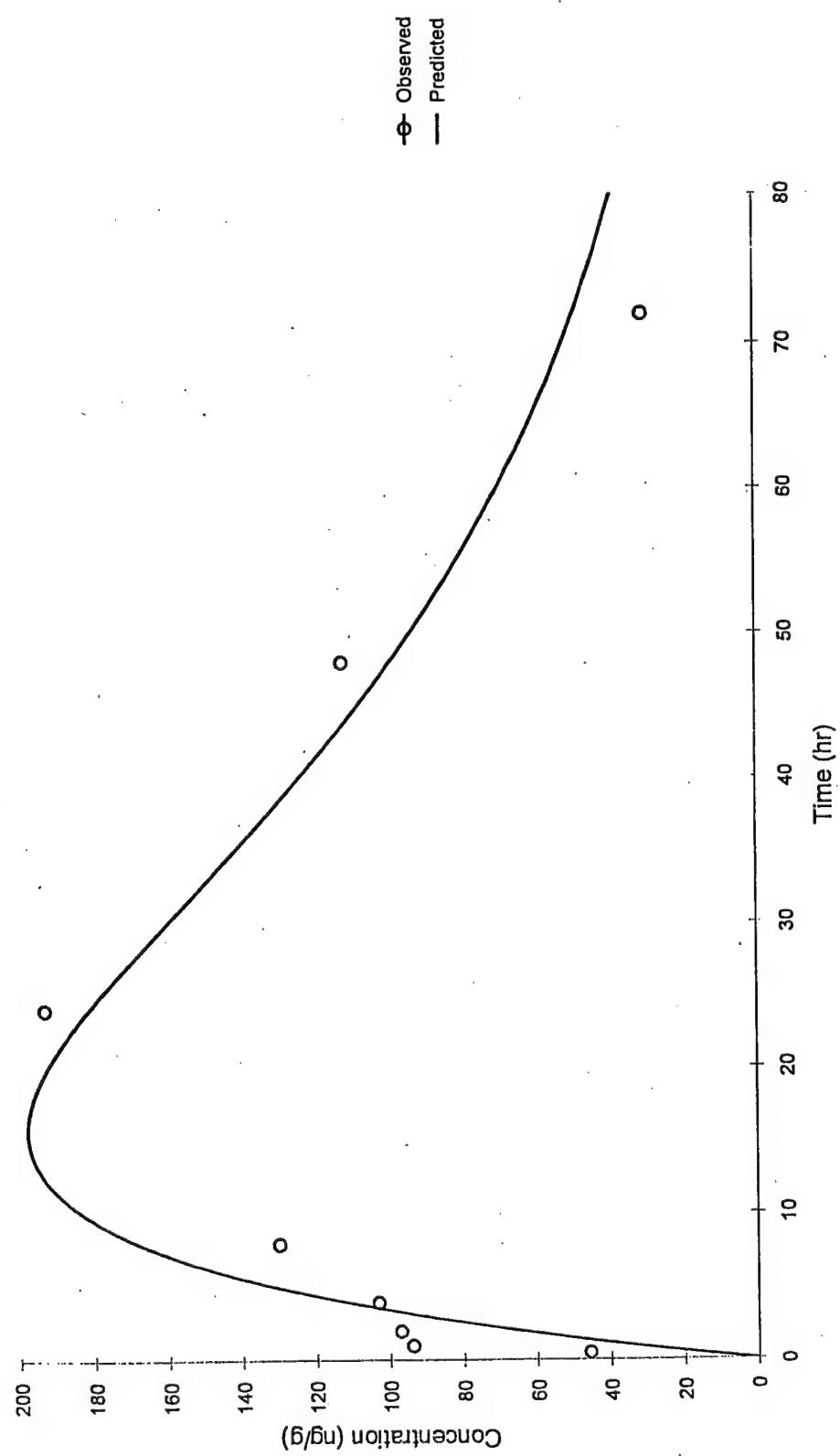


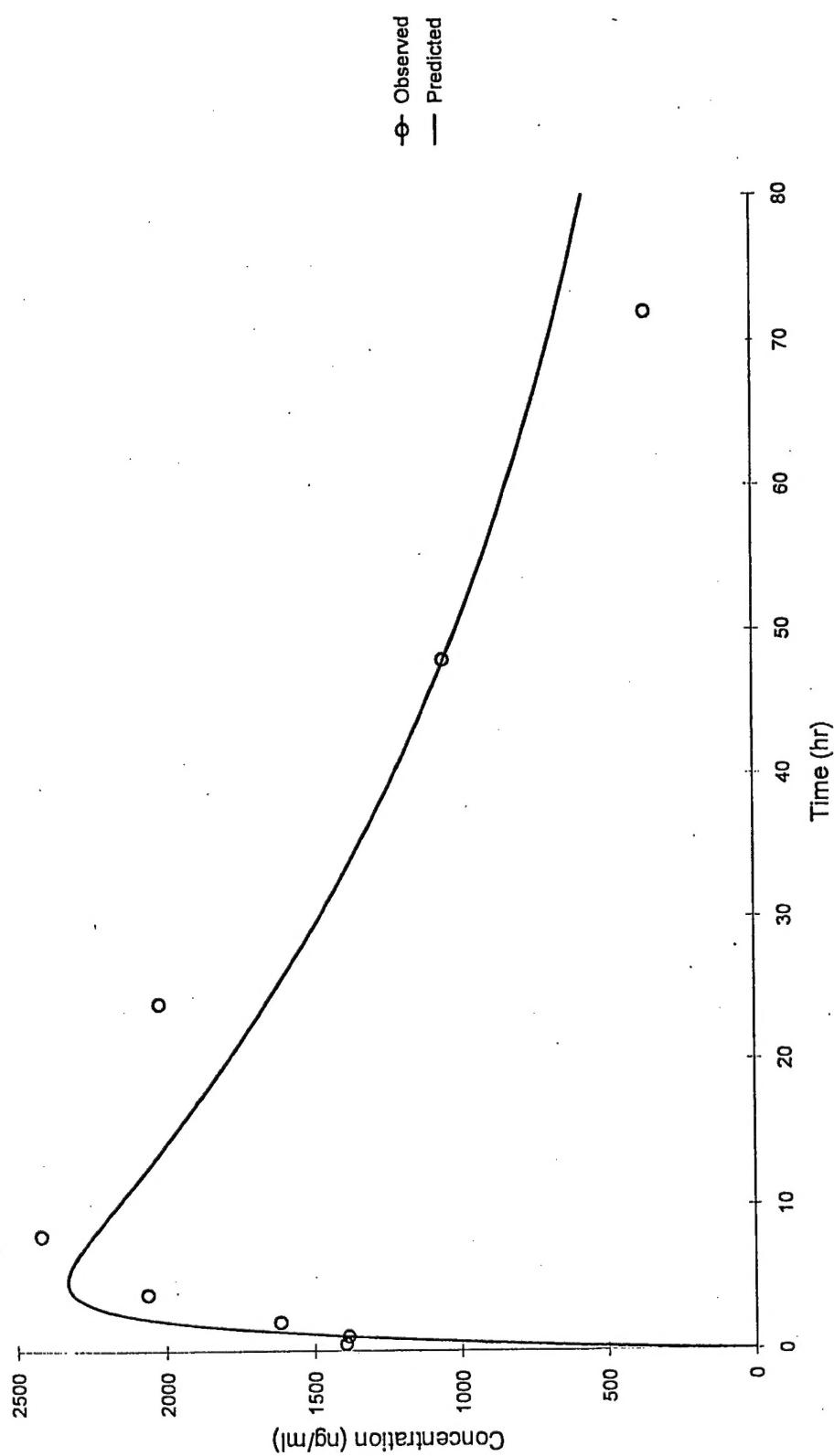


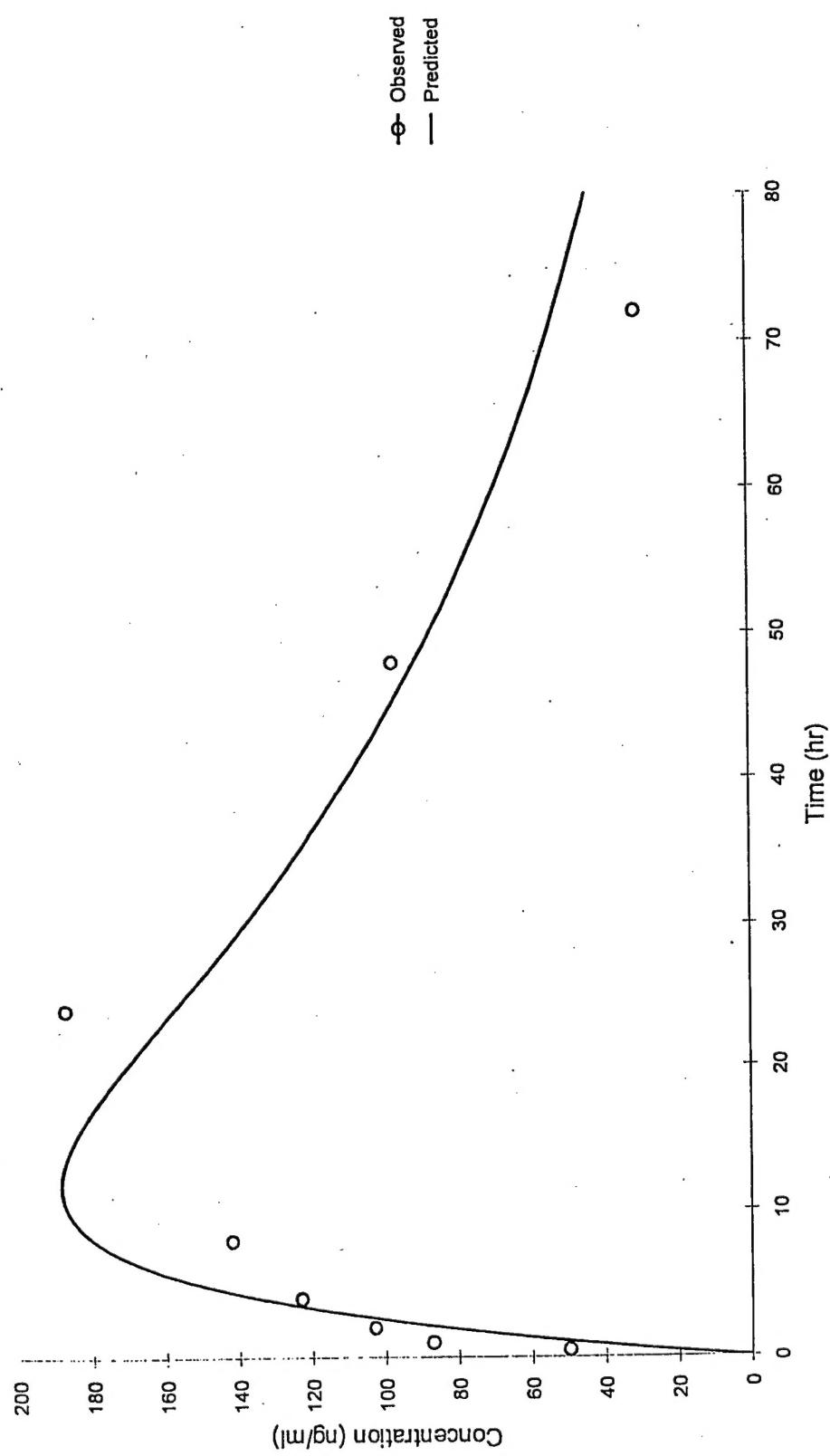












Conclusions

The results of studies presented in this report support our hypothesis that simultaneous exposure to pyridostigmine bromide (PB), *N,N*-diethyl-*m*-toluamide (DEET), and permethrin can potentiate their toxicity (Study #1). Combined daily dermal exposure to doses low enough to be representative of human exposure levels, i.e., 40 mg/kg DEET and 0.13 mg/kg permethrin for 60 days, caused neuronal cell death in the motor cerebral cortex, the dentate gyrus, the CA₁ and CA₃ subfields of the hippocampus, and the cerebellum. Neuronal loss was also corroborated by a significant decrease in microtubule associated protein -2-immunoreactive elements. These alterations were also accompanied by increased glial fibrillary acidic protein (GFAP)-immunoreactivity, indicating significant hypertrophy of astrocytes. Histopathological alterations were quantified by counting the number of surviving and dead cells. The investigators carrying out the counts were unaware of the identity of the sections, and the order of examination of the sections was randomized.

Although the mechanisms by which test compounds induce neuronal lesions are not known, there is evidence that oxidative stress may play a major role in this process. First, brain regions that exhibited neuropathological lesions, i.e., motor cortex, somatosensory cortex, CA₁ and CA₃ subfield of the hippocampus, purkinje cells of the cerebellum, are all known to be vulnerable to oxidative stress. Second, to explore this mechanism we investigated the possibility that test compounds cause the production of reactive metabolites that lead to oxidative stress. The results indicated that dermal exposure to DEET and permethrin increased the urinary level of 3-nitrotyrosine, a marker for oxidative stress (Study #2). Also, dermal application of DEET and permethrin significantly increased the release of brain mitochondrial cytochrome c, that has been reported to correlate with the induction of apoptosis process (Study #3). These results support the suggestion that the mechanism by which test compounds cause neuronal damage involves the production of free radical species resulting from excessive generation of reactive oxygen species (ROS) or by decreased defensive mechanisms against oxidative damage. These results may contribute to understanding enhanced toxicity following exposure to test compounds.

During the reporting period, we continued our studies into the effect of test compounds on the liver, by investigating the effect of test compounds on hepatic cytochrome CYP450. The results show that dermal application of DEET, alone or in combination with permethrin, increased urinary excretion of 6 β -hydroxycortisol in rats, a biomarker of hepatic cytochrome P450 CYP3A induction (Study #5).

We have also investigated the bioavailability, pharmacokinetics, and metabolism of DEET and permethrin when applied dermally, alone or in combination. The results showed that dermal bioavailability of a single dose of DEET was increased following combined exposure with dermal permethrin. On the other hand, combined exposure to dermal DEET decreased the bioavailability of dermal permethrin.

Appendices

Published Papers

1. Abdel-Rahman, A.A., Shetty, A.K., and Abou-Donia, M.B. (2001). Sub-chronic dermal applicaton of *N,N*-diethyl-*m*-toluamide (DEET) and permethrin to adult rats, alone and in combination, causes diffused neuronal cell death and cytoskeletal abnormalities in the cerebral cortex and the hippocampus, and Purkinje neuron loss in cerebellum. *Exp. Neurol.* **172**: 153-171.
2. Abu-Qare, A.W., and Abou-Donia, M.B. (2001). DEET (*N,N*-diethyl-*m*-toluamide) alone and in combination with permethrin increased urinary excretion of 6 β -hydroxycortisol in rats, a marker of hepatic CYP3A induction. *J. Toxicol. Environ. Health Part A*, **64**: 373-384.
3. Abu-Qare, A.W., Suliman, Hagir B., and Abou-Donia, M.B. (2001). Induction of Urinary Excretion of 3-nitrotyrosine, a marker of oxidative stress, following administration of Pyridostigmine bromide, DEET (*N,N*-diethyl-*m*-toluamide) and permethrin, alone and in combination in rats. *Toxicolgy Letters*, **121**: 127-134.
4. Abu-Qare, A.W., and Abou-Donia, M.B. (2001). Combined exposure to DEET (*N,N*-diethyl-*m*-toluamide) alone and in combination with permethrin-induced release of rat brain mitochondrial cytochrome c. *J. Toxicol. Environ. Health Part A*, **63**: 243-252.

Manuscript Submitted

1. Abdel-Rahman, A.A., Shetty, A.K., and Abou-Donia, M.B. (2002). Disruption of Blood Brain Barrier and Neuronal Cell Death in Cingulate Cortex, Dentage Gyrus, Thalamus, and Hypothalamus in a Rat Model of Gulf War Syndrome (Submitted for Publication).

Abstracts

To be presented in the SOT Annual Meeting – March 2002

1. Abu-Qare, A.W., Abdel-Rahman, A.A., and Abou-Donia, M.B. (2002). Pharmacokinetic Interactions between Pyridostigmine Bromide (PB) and Permethrin in Rats.
2. Abdel-Rahman, A.A., Shetty, A.K., and Abou-Donia, M.B. (2002). Neuron Degeneration induced by subchronic application of *N,N*-diethyl-*m*-toluamide (DEET) and Permethrin, alone and in combination to rats.